CO-EXTRA

GM and non-GM supply chains: their CO-EXistence and TRAceability

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CO Confidential, only for members of the consortium (including the Commission Services)	

1 Summary

This deliverable is a status report on some of the results of activities in several tasks, where the focus is to identify the possibilities and limitations of target amplification methods alternative to PCR in combination with signal amplification technologies. The involved tasks are mainly T6.5, but also T6.1, T6.2, T6.3 in workpackage 6 and T5.10 in workpackage 5. The nature of the deliverable is described as a “public” intermediary report. The purpose of the report is to give an overview of achievements and limitations of the different methods in order to assist the project partners to critically evaluate the key aspects of the current activities.
The preparation of this report was coordinated by Lillian Roth, Jutta Zagon and Hermann Broll from the Federal Institute for Risk Assessment (BfR), Berlin, Germany (partner 11).

September 17th 2007

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Table of contents

2 Background

In a global perspective there are now many genetically modified (GM) crops that have been approved for commercialization. The planted area of GM plants increased 60-fold between 1996 (1.7 million hectares) and 2006 (102 million hectares), and mainly soybean, maize, rape seed and cotton varieties are grown (James 2006). GM plants for human consumption or animal feed are grown on a large scale in the USA, Argentina, Canada, Brazil, China and India. The acceptance of this technology does not follow the traditional dichotomy between the developed vs. developing countries. Whilst GM food is readily accepted in the USA, European consumers have shown considerable reluctance due to a lack of confidence in food safety following previous food crises (Eurobarometer, 2005).

There are several regulations, requirements and rules established in Europe in order to enable the consumer to make an informed choice and facilitate fairness of transactions between seller and purchaser regarding GM foods and feeds.

Regulation (EC) No 178/2002 (EC, 2002), known as the General Food Law, lays down the general principles and requirements of food law, establishes the European Food Safety Authority and lays down procedures in food safety matters. Article 18 of this Regulation also contains general rules regarding traceability, saying that traceability of food, feed, food-producing animals and any other substance intended to be, or expected to be, incorporated into a food or feed, must be established at all stages of production, processing and distribution. This Regulation establishes the principle that the primary responsibility for ensuring compliance with food law is up to the food business.

Regarding GM organisms (GMOs), there are more specific regulations concerning the marketing and traceability. Regulations (EC) No 1829/2003 (EC, 2003a) and No 1830/2003 (EC, 2003b) describe the labelling and traceability rules with respect to GMOs. Products which are, consist of or contain GMOs need to be labelled and traceable in each stage of production, processing and distribution. Only where the GMO content of an ingredient of a mixed product is below 0.9%, no labelling is required, provided that these trace amounts are adventitious or technically unavoidable, and that the GMO in question has been authorised for food/feed use in the EU. This rule is also applicable for food and feed products which do not contain any newly introduced DNA or protein due to rigorous processing like refined oils or products derived from hydrolysed starch.

In principle, GMOs can be identified by detecting either the inherent material (DNA), the mRNA transcribed from the newly introduced gene or the resulting protein, metabolites or phenotypes, respectively. But in practice, the assays on raw materials as seeds are generally carried out with the polymerase chain reaction (PCR) or with immunological assays such as the enzyme-linked immunoassay (ELISA). Irrespective of a variety of potentially available methods for DNA analysis, so far only PCR in its different formats has found broad

application in GMO detection/analysis as a generally accepted method to test for regulatory compliance.
GMO quantification is most accurately done by using real time PCR. However, this requires sophisticated equipment, dedicated, strictly separated laboratory areas and well trained and experienced personnel. Carrying out such an analysis is time consuming and cost intensive. So far, individual reactions need to be run for each single event, in order to prove its presence in the sample under investigation. One possibility to overcome this bottleneck is to combine several PCR systems and to apply them in a multiplex procedure. However, it has not been shown to be applicable in routine analysis for more than a few primer systems so far. This might be due to the limited amounts of reaction components, such as dNTPs after some cycles and the competition between the several primer systems. It has been experienced that for some specific PCR primer sets a relative limit of detection (LOD_{rel}) below 0.1 % was not feasible.

This document will describe the state-of-the-art within the Co-Extra project of alternative target amplification and signal amplification methods alternative to PCR, with a focus on multiplexing and integration with e.g. array technology.

3 Specific contributions from the partners

3.1 Target amplification methods

3.1.1 NASBA (NIB, partner 6)

Principle:

Basically, in the WP6 framework we are developing a new target amplification procedure based on NASBA (a transcription based amplification as an alternative amplification method to PCR), but including also several innovative molecular biology improvements. This target amplification method is aimed for GMO detection in multiplex process by hybridizing its amplification products on microarray for detection, identification (Task 6.1) and also quantification (Task 6.2) of GMO targets. In parallel, we are also adapting the process for real-time detection of the target DNA (T5.10).

Possible applications:

The first application is designed for multiplexed GMO semi-quantitative analysis on low density microarray. We think that beside GMO detection, this process could be used in combination with any detection DNA microarrays where the sensitivity is a problem. Additional improvement over PCR based target amplification is speed of analysis and possibility of semi-quantification on the microarray (up-to-now, with PCR only qualitative results can be obtained, unless sub-sampling strategies are used for determining the GMO content position versus a predefined threshold).

Detailed description of the procedure:

The amplification process consists of a first step (named template synthesis) in which the extracted DNA (sample) is amplified in a single cycle with DNA polymerase (for instance Taq polymerase) with bipartite oligonucleotide primer pairs. For each primer pair, one primer is composed of a specific sequence complementary to the target DNA to be detected and one sequence corresponding to the RNA polymerase T7 promoter DNA, on the 5'-end, while the second bipartite primer is composed of a specific sequence complementary to the target DNA to be detected and one sequence corresponding to another none related sequence (here the RNA polymerase SP6 promoter DNA), on the 5'-end. The result of the template synthesis is a short double stranded DNA fragment corresponding to the target DNA to be detected and framed by the RNA polymerase T7 promoter DNA on one edge, and the RNA polymerase SP6 promoter DNA.

In the second step of the process, the newly synthesized template (diluted) is amplified using the NASBA (Nucleic Acid sequence-based amplification) method to obtain cRNA. Instead of using two specific primers (one also harbouring the T7-promoter sequence) as it is described in the NASBA process, we simply use "universal primers" corresponding to the T7-promoter and the SP6-promoter sequence. Moreover, the extension time is controlled to stay in the linear range of the amplification for quantitative purpose.

In the case of multiplex detection and quantification on microarray platform, the use of modified nucleotides (such as aminoallyl-UTP) is required for indirect labelling of the products (e.g. with Cy-5 and Cy-3 dyes) for visualization of the bound products on the array.

Current results

Singleplex reactions

The development stages first focused on two important screening elements for GM maize: the invertase (IVR) endogene which is present in every maize line (conventional or GM), and the cauliflower mosaic virus (CaMV) 35S promoter (P35S) which is the most common element used in transgenic construct (41% of all existing constructs (BATS report 2003), more than 73% of EU authorized GM in food and feed and close to 90% of EU authorized transgenic maize in food and feed (EC 2007)). These target elements were amplified in simplex NASBA reactions showing up-to a 22 Ct-difference (more than $4x10^6$ fold amplified) when compared to the control DNA on real-time PCR. The method was shown to be sensitive since less than 5 ng of genomic DNA were needed to obtain solid amplification of the target DNA. Moreover, the amplification was shown to be specific: only the target DNA is amplified.

Several improvements were introduced to the original NASBA procedure, including primer design, salt composition and extension time of the amplification. Eventually, in presence of improved primers, the NASBA extension time was dramatically reduced from 3 hours to 15-25 min. Under these conditions, NASBA showed equal amplification efficiency for both P35S and the maize invertase (*ivr*) elements with good amplification rate (18-Ct difference when compared to control for both elements after 25 min. amplification). Moreover, we showed that within 5 to 25 min of NASBA extension, the amplification is in a linear phase compatible with quantitative analysis of DNA contents. More recently, new NASBA amplicons were designed for specific detection of the Mon810 event specific maize line and for the *Agrobacterium tumefaciens* derived nopaline synthase terminator (t-NOS) screening element. As for P35S, t-NOS is one of the most commonly used elements in transgenic construct (31% of all known constructs (BATS 2003), 63 % of EU authorized GM in food and feed and 55% of EU authorized transgenic maize lines in food and feed (EC 2007)). Similar results were obtained for Mon810 event-specific and t-NOS amplicons when compared to IVR and P35S amplicons in term of specificity, sensitivity and amplification rate.

Linearity of the singleplex reactions was also assayed for each amplicon by varying the copy numbers of the targets (serial dilutions) and the transgenic content in the maize sample (from 100% to 0.1% of transgenic line). These assays showed that singleplex NASBA is semi-quantitative for a wide range of relative GMO concentrations (100% to 1%) and for a wide range of absolute concentrations (60000 to 50 copies).

- Linear and robust singleplex amplification was observed with the customized NASBA conditions for IVR maize endogene, P35S and t-NOS screening elements and Mon810 event-specific element. Same amplification efficiency is obtained for all markers. The experimental time was substantially reduced to 15-25 min.

Duplex reactions

Once the NASBA procedure was set for single target amplification, duplex NASBA reactions were performed targeting the same P35S and *ivr* elements. Similar amplification rates were found for both target elements in duplex and they correspond to the amplification observed for each singleplex NASBA reaction. These results demonstrate the specificity of the first amplification step, and the non-discriminatory property of the “universal” primer set for any target element. Duplex amplification is also linear from 5 to 25 min of extension, which makes duplex NASBA suitable for quantitative analysis of DNA contents.

Finally, we also determined the limits of detection (LOD) and quantification (LOQ) of singleplex and duplex NASBA for both elements. NASBA was able to amplify both targets with as low as 15 starting copies while the LOQ was determined to 50 copies. Moreover, as for the singleplex reactions, we showed that NASBA duplex is suitable for semi-quantitative detection for maize material with 100% to 1% of relative GM concentration and with 60,000 to 50 copies of the targeted element (absolute concentration).

- Linear and robust duplex amplification was observed witht the customized NASBA conditions (for IVR and P35S markers). In duplex NASBA, amplification

efficiency of each marker is comparable to its singleplex amplification. The same amplification efficiency is obtained for all markers in duplex NASBA. Sensitivity is suitable for on-chip multiplex screening.

Tri (3)-plex and tetra (4)-plex reactions

Triplex reactions were assayed for the following combinations: *ivr*, P35S and Mon810 event-specific and *ivr*, P35S and t-NOS. Once again comparable amplification rates were found for each amplicon in regard to the singleplex reactions. Moreover, all three amplicons behaved the same in the triplex reaction making it semi-quantitative for a 25 min extension time.

As for duplex and triplex reactions, NASBA triplex was found suitable for semi-quantitative detection for maize material with 100% to 1% relative GM concentration and 60,000 to 50 copies of the targeted element (absolute concentration).

When assayed, a tetra-plex reaction including all four amplicons (*ivr*, P35S, t-NOS and Mon810 event-specific) failed to show semi-quantitative amplification. The IVR, 35S and t-NOS behaved the same when compared to triplex, duplex and monoplex reactions. However, Mon810 event-specific amplicon showed a drop in the observed amplification rate. This lead us to re-design the assay for tetra-plex semi-quantitative amplification (in process).

The different characteristics of our customized NASBA (linear and equal amplification of amplicons in singleplex, duplex and triplex reactions, sensitivity, specificity, short extension time, single strand final product, LOD and LOQ) are suitable for the development of our amplification platform for on-chip (microarray) detection. We are currently testing the usability of NASBA for the detection of the Mon810 GM maize line on a customized microarray designed by the Institute of Food Safety (RIKILT) from Wageningen, The Netherlands (partner 9).

- In triplex NASBA, amplification efficiency of each marker is comparable to its singleplex amplification. The same amplification efficiency is obtained for all markers in triplex NASBA. Attention must still be paid to the interaction between primers in the case of multiplex amplification. The observed performance meets the requirements for multiplex screening on array.

We are currently testing triplex screening (Mon810 event specific, *ivr* and P35S) on a low density array designed by RIKILT (partner #9) for Mon810 and RRS detection.

Future plans

Extension of the multiplexing

In a first step, new primers will be designed to extend the spectrum of GM maize detection with NASBA. For this purpose, NASBA primers were designed for specific detection of Bt176, Bt11, T25, Mon863, GA21, NK603 and TC1507 maize lines, all of these being authorized in Food and Feed under EU legislation (EC 2007). Also, some other screening elements (such as CaMV 35S terminator [T35S], *Bacillus thuringiensis* derived *Cry* genes, or a 5-enolpyruvylshikimate-3-phosphate synthase [*EPSPS*] gene) present in several transgenic constructs will be targeted by new NASBA amplicons as well as the CaMV control to discriminate if the presence of P35S element is due to the transgenic construct or contamination with the CaMV. Once the singleplex NASBA reaction will have been set up for linear amplification of each element with comparable amplification efficiency with the elements P35S, *ivr*, t-NOS and Mon810 (event-specific), the number of elements simultaneously amplified by NASBA will be sequentially increased.

On-chip detection

Our preliminary results for on-chip detection on RIKILT microarray lead us to pay more attention to the target signal. Our customized NASBA is normally performed in presence of amino-allyl UTP (aa-UTP) to allow indirect labelling of the cRNA products. We are currently testing the

possibility to perform a double-labelling on cRNA and cDNA products (the latter being an important by-product of NASBA amplification) using aa-UTP and amino-allyl dUTP (aa-dUTP). This double labelling should increase the signal level for better on-chip detection of GMO. Other signal detection systems could also be assayed to ensure a good signal/background ratio.

In order to have a support for the multiplex detection of GM maize lines (and GTS-40-3-2 [Roundup Ready soybean; RRS]), we are currently collaborating with another CoExtra partner Eppendorf Array Technology (EAT, partner #31), Namur, Belgium. This partner has already developed a "DualChip" for the microarray-based detection of screening elements present in several GMOs, such as RRS, Bt11, GA21, Mon810, T25 and Bt176. This partner is currently producing a customized low-density microarray suitable for semi-quantitative GMO detection of NASBA multiplex products (IVR, 35S, t-NOS and Mon810 event-specific amplicons). The probes are designed by EAT in order to match with the NASBA amplicons, and tested both *in silico* and experimentally.

Conclusions on the NASBA model

We adapted the traditional NASBA protocol to be compatible with multiplexing DNA-based analyses of GMO contents with a good amplification rate. The NASBA products can be hybridized on microarray support leading to a high throughput analysis system for target detection as the system is sensitive enough. In our hands, the customized NASBA protocol was found to be reliable for the detection of GMO targets with good specificity and sensitivity. The amplification of our customized NASBA appears to be linear and sufficient for on-chip detection. This finding complies with the need for a semi-quantitative analysis tool for GMO content analysis. The limit of quantification was found to be compatible with on-chip detection procedure.

On an economical point of view, because of the provider's monopoly on NASBA kit, the use of NASBA as alternative to PCR is more expensive (32.4€ per singleplex reaction including the use of modified nucleotides for on-chip detection but not including the DNA extraction and the coupling-hybridization costs). The price rapidly decreases with the number of "plexing" used in the reaction (3.3€ for a 10-plex). A solution to avoid the high price of NASBA kit would be to develop an in-house NASBA mix (planned in further experimentation steps).

In conclusion, NASBA has a good potential as new target and signal amplification method alternative to PCR, especially as it enables semiquantitative multiplex analysis. However, some improvements still need to be done regarding the number of amplicons used in a same reaction, the price of the method, hybridization and detection of signal on the microarray. The degree of multiplexing vs. use of parallel oligoplexing is performance dependent, but a high degree of multiplexing is preferred (less labour).

3.1.2 MQDA-PCR (Matforsk, partner 29)

Finalising designing primers and probes and optimising the PCR procedure

We have finalised the redesign of different primers and probe sets for a 9-plex PCR covering Mon810, T25, Ga21, TC1507, NK603, Mon863 (all event-specific), and Bt11 and Bt176 (both construct specific) and a maize reference gene. All template DNAs originate from GMO CRM material (maize powder) or are from maize samples from other sources. In the 12-24 month period we have been optimising the 2-step PCR procedure. Different treatments of the samples after the first (6 cycle) PCR step gave different results. Methods degrading bipartite primers or polymerases have been tested. The best solution is tryptic inactivation of the DNA polymerase after the first PCR. Primer and probe concentrations have been optimised. Control experiments omitting the head primer in the second PCR gave very weak signals showing that the head primer performs the amplification. This is essential for the quantitative aspect of the assay. Quantitative experiments using mixtures of 8 GMO maize templates were carried out. The content of Mon810, Mon863 and TC1507 was varied (from 0 to 2%) while keeping the others constant (different constant levels for different constructs 0.1 to 4 %). Amplifications were done in triplicates. Clear quantitative signals were detected. R^2 coefficients varied from 0.80 to 0.98 depending on the procedure and the event. Signals from the events that we used at a fixed amount did not vary. 0.1% GMO was easily detected. Background was low. A designed experiment with the 8 GMOs showed marginal influence of each GMO on the signals of other GMOs and little influence on specific GMO signals in presence of total GMO up to 7 %. Currently we are comparing the method with results from singleplex real time PCR.

3.1.3 Ligation-mediated amplification system/ MLPA (Matforsk)

Multiplex PCR is feasible in the 10–plex range. For higher multiplexing other methods may be of interest. The ligation mediated amplification system MLPA has been reported to work in a 40 plex system. We are developing an alternative system for detection of the above 8 maize events using ligation-mediated PCR. Ligation-probes were event-specific. Ligation and PCR was according to the MLPA procedure. Amplicons were detected by capillary electrophoresis. So far a 5 plex system for detection of Ga21, NK603, Bt11, Mon810 and Mon863 has been developed. Some variability in sensitivity has been observed, partly, but not solemnly due to the large maize reference signal compared to GMO signals. More GMO events will be included. The method must be optimised to increase sensitivity. Background is low.

3.1.4 Multiple Displacement Amplification/ MDA (BfR, partner 11; NVI, partner 7)

BfR

In order to avoid any construct- or event-specific amplification steps in GMO detection, the BfR uses whole genome amplification by multiple displacement amplification (MDA) as a tool to provide large amounts of sample DNA. Sufficient amounts of high quality sample DNA should allow subsequent identification of target DNA by direct hybridisation of the amplified DNA (mdaDNA) to probes specific for individual GMOs.

The MDA technique is based on the use of the phi29 DNA polymerase from bacteriophage phi29 and random hexamer primers for the efficient and accurate amplification of genomic DNA. Defining properties of phi29 DNA polymerase are a strong strand displacement activity and a high processivity, leading to the synthesis of large amounts of DNA with fragments in the size >70 kb (Blanco et al., 1989) and favouring a genome-wide uniform representation of gene loci. A 3'→5' exonuclease activity associated with the enzyme, results in error rates between 10-5 and 10-6 (Esteban et al., 1993) compared with an error rate of about 10-3 generated by DNA Taq polymerase in PCR (Dunning et al., 1988). Small quantities such as 1 ng of genomic DNA template introduced into the reaction are sufficient to produce microgram yields of high molecular weight DNA in an overnight isothermal reaction (Luthra & Mediros, 2004; Blanco & Sulas 1996). The mechanism of phi29 polymerase amplification reaction is shown in Figure 1.

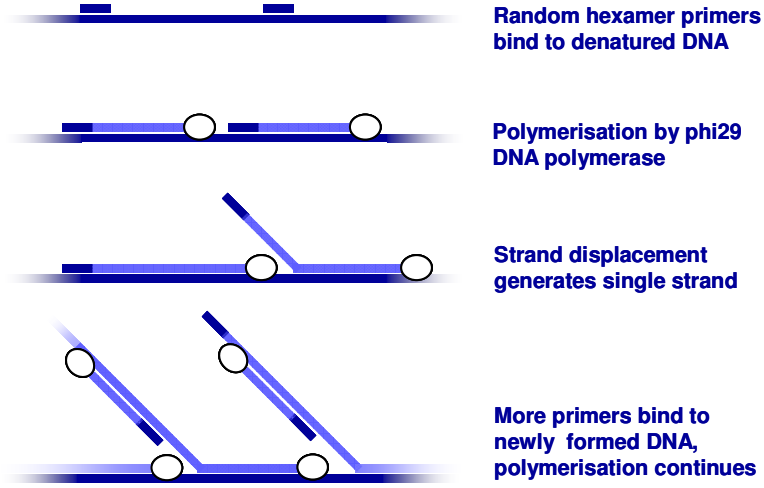

Figure 1. DNA amplification by phi29 DNA Polymerase. Binding of hexamer primers, polymerisation and strand displacement.

We optimized the MDA procedure for the amplification of genomic DNA of maize lines Event 176 (Bt176, Syngenta Seeds), Mon810 and Mon863 (Monsanto). Different Real-Time PCR systems were used for an evaluation of MDA efficiency and proportion of amplification over the genome. Therefore we selected different maize specific single-copy gene targets: alcohol dehydrogenase 1 (adh1)(ISO, 2005), high mobility group protein (hmg) (ISO, 2005), invertase (inv) (Ronning et al., 2003) and the following GMO specific target genes: cry3Bb1 for Mon863 (Monsanto, 2004), cry1Ab for Bt176 (ISO, 2005), mail for Mon810 (ISO, 2005).

Two commercial MDA kits (GenomiPhi Kit from GE Healthcare and RepliG Kit from Qiagen) and a single component reaction set-up were tested. Quantification of mdaDNA was accomplished by fluorometric and spectrophotometric measurement. Quantitative results indicate the generation of single stranded (ss)DNA in addition to double stranded (ds)DNA. Total mdaDNA yield was calculated by a combination of fluorometry (measures dsDNA) and spectrophotometry (measures total DNA) results. Irrespective of the amount of starting material, total DNA yield averages about 6 µg of total mdaDNA with approximately 25-30 % ssDNA (GenomiPhi Kit). The average total mdaDNA yield from no template control (NTC) reactions is similar to the amounts of DNA samples, but the dsDNA share is distinctly lower (65 % ssDNA for GenomiPhi Kit). As the proportion of ssDNA in mdaDNA increases in NTC samples and in samples with extremely

low DNA input, a large fraction of ssDNA might be a sign of de novo synthesis of unspecific DNA, presumably derived from primer extension.

Real-Time PCR was used for the determination of MDA efficiency for the absolute amplification of target specific DNA. As MDA is an endpoint reaction due to the limited amounts of individual reagents, the amplification efficiency shows a negative correlation to the amount of starting material. By expanding the reaction volume, absolute specific mdaDNA yield and amplification efficiency can be increased. In our laboratory we used standard reaction volumes according to the kit manufacturers' instructions. The amplification yield for specific target DNA was calculated by comparing Real-Time PCR Threshold-Cycle (Ct) differences for adh1 system of gDNA to mdaDNA obtained in reactions with different amounts of starting material. From each reaction, an identical volume was introduced into TaqMan™-PCR and was compared to the corresponding amount of starting gDNA. For an MDA reaction with a starting amount of 172 haploid maize genome copies the quantification of mdaDNA by standard curve calibration demonstrates a 17000 fold amplification of maize specific DNA. With increasing starting amounts, amplification efficiency decreases. In NTC samples, no target specific DNA was generated by MDA.

For application of MDA in GMO analysis, it is necessary that gDNA quality is preserved after MDA in order to detect the target of choice. For exclusion of a potential amplification bias by MDA, we examined several randomly selected target sequences for their proportional representation in mdaDNA compared to gDNA.

The proportional representation of amplified targets after Real-Time PCR was evaluated by comparing the copy numbers of different genes for a given mass of input DNA. Applying several primer/probe-systems targeted to maize specific adh1, hmg and inv genes and GMO specific cry3Bb1, cry1Ab and mail genes, respectively, showed a largely unbiased amplification when using a commercial MDA kit. The maximum bias of amplification we observed is a 3.5-fold over-representation of the mail gene. This corresponds to results for maximum bias described in literature (Pinard et al., 2006; Dean et al., 2002; Hosono, 2003) and shows that the MDA amplifies the genomic DNA representatively.

Multiple displacement amplification provides the means to generate large amounts of DNA from small amounts of GMO sample material in a simple, isothermal reaction. The resulting mdaDNA is of high quality and can directly be applied to subsequent detection methods for GMO analysis.

NVI

For the microarray-based methods developed at the NVI, two different strategies have been used to prepare DNA for hybridisation: 1) fragmentation and end-labelling of total DNA (see section 3.2.4), and 2) DNA preparation using multiple displacement amplification protocols. Genomic DNA has been used directly when sufficient material has been available. For experimental situations where adequate amounts of DNA could not be acquired, a commercial kit for multiple displacement amplification (REPLI-g technology; QIAGEN AB) was used. With small amounts of DNA extracted from rice leaves (<100ng), it was possible to acquire sufficient DNA for array hybridizations with standard protocols (several µg).

The principle of multiple displacement amplification was also tested with the objective to perform a more targeted amplification of genetic loci relevant for GMO testing. In a standard multiple displacement amplification reaction, random nucleotide oligos (hexamers) are added and the strand displacement polymerase (phi29) will attempt to amplify the entire template DNA in an unbiased fashion. As an alternative phi29 DNA polymerase was combined with sets of specific oligos, targeting genes of particular interest. The idea was to see if small sets of specific oligos were able to generate preferential amplification of genetic elements relevant for GMO testing (in a multiplexing context). If this approach had been successful it could represent a fast, isothermal way of screening for the presence of a potentially large number of different GM elements. With a model system building on Arabidopsis thaliana different combinations of oligos and experimental setup were explored, but unfortunately without significant amplification of the targeted genomic regions.

3.1.5 Loop-Mediated Amplification/ LAMP (Lumora, partner 28)

Loop-Mediated Amplification (LAMP) was first described by Notomi *et al.* (2000), and has subsequently been cited in over 100 publications. LAMP is characterised by the use of primers capable of forming loops. LAMP isothermally amplifies DNA targets with high specificity even without confirmatory probes. This is a reflection of the amplification mechanism which requires four independent target sites to be recognized for amplification to occur, compared to just two in PCR (Mori *et al.*, 2001; Nagamine *et al.*, 2002). LAMP has now been demonstrated with a wide variety of different targets from different matrices, including the detection of GMO (Fukuta *et al.*, 2004). LAMP has become a very well characterized method with high specificity and sensitivity and has been validated for diagnostic products. LAMP is one of a number of isothermal amplification technologies that can be interfaced with Lumora's reporter system BART (bioluminescent assay for real-time: see Section 3.2.2).

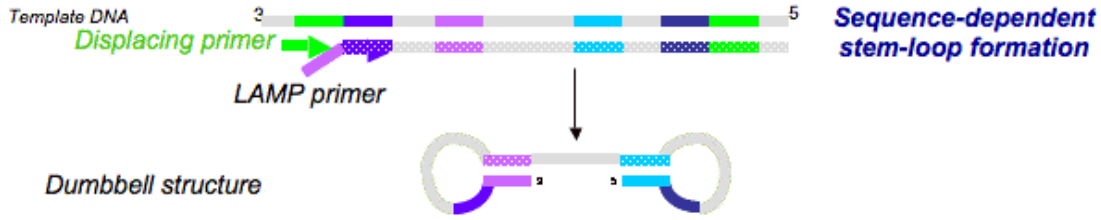

Figure 2. Basic mechanism underlying LAMP.

Primers were designed to the 3' junction of the GTS40-3-2 Round-up Ready Soya insert. The final sequences used in the detection protocol are as follows:

B1cB2 cat gct gtt gcc tat tag ggt c tttt gat taa gct tca aca tgt gaa gg (loop forming primer)
B3 ctc ctg cta tta cat gag (displacing primer)
LoopB gta gta cac tca cca gt (loop primer : additional accelerating primer binding loop)
F1cF2 ggg atc gga gaa gaa ctg ttt g tttt ctt ctt cac gaa ctt ctc gac (loop forming primer)
F3 gag aag gta gtt ctc ttc (displacing primer)
LoopF gaa tgg cct ggt cgt cg (loop primer)

These primers are spatially associated with the target site as follows:

5' ---[B3][B2]-[LoopB]-[B1]----------
junction: |
3' ----------[F1]-[LoopF]-[F2][F3]----

Specifically (see next page):

```
CAGCAATTTG AATATTAAGT AACTGCTTCT CCCAGAATGA TCGGAGTTTC

  [       B3        ]                                [
TCCTCCTGCT ATTACATGAG CAAAAATAAA AAATAAATAA AAGATAAGAT

       B2           ]  [     Loop B     ][        B1
TAAGCTTCAA CATGTGAAGG AGTAGTACAC TCACCAGTGA CCCTAATAGG

          ]
CAACAGCATG AAAAAAAATA AAAAAGAATA AAAATAGCAT CTACATATAG

                    Junction:|   [      F1
CTTCTCGTTG TTAGAAAAAC AAAACTATTT GGGATCGGAG AAGAACTGTT

 ]    [     Loop F      ]          [          F2
TGAGGCGAAT GGCCTGGTCG TCGCGGCCAT CGTCGAGAAG TTCGTGAAGA

 ]             [      F3          ]
AGCTCGAATG CGGTGAGAAG GTAGTTCTCT TCCAACAGAA AGTTCACCAC
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DNA was extracted from 50mg flour of Roundup Ready Soya from Sigma (Fluka # 94162, Lot # 1129490) using a Promega Wizard genomic DNA purification kit (A79). The following materials were extracted: 0%, 0.1%, 0.5%, 1%, 2% & 5%.

Results analysed by agarose gel electrophoresis show that the GM event 40-3-2 is amplified in all the standards containing the event, but not in uncontaminated material. Further analysis was carried out using the BART assay (see Section 3.2.2) for both 40-3-2 and, together with partner NIAB, for events RF3 and MS8 of Bayer InVigor canola corresponding to the two parental lines of the hybrid grown.

3.1.6 Padlock probe ligation (RIKILT, partner 9)

RIKILT has explored the padlock approach for multi-detection of (unapproved) GMOs. The padlock approach involves ligation detection, which can be done in multiplex, followed by universal PCR amplification of positive products and subsequent identification on a universal microarray.

Introduction

The padlock approach (Figure 3) is based on the principle that a unique sequence is first detected by a so-called padlock probe in the pool of genomic plant DNA. Only when both ends of the padlock probe can hybridize to their specific complementary target sequence, ligation can occur and this will result in a circular molecule. This method can be carried out in multiplex with up to 1500 padlock probes in a single reaction as is already being done for SNP detection in human settings (Hardenbol *et al.*, 2003).

After ligation, universal primer sites in the padlock probe enable amplification and Cy3 labelling of all circularized padlocks, while linear probes will not result in exponentially amplifiable products.

Identification of different products occurs on the basis of microarray hybridization. Each padlock probe contains a unique ZIP code, a random 20 nucleotide sequence, with similar hybridization conditions for different ZIP codes. Only amplified padlock probes will result in a significant signal when the pool of PCR products is hybridized to a microarray with complementary ZIP codes.

For testing purposes of newly developed probes, detection can also occur with real time PCR, either with the SYBR green fluorescent dye, or a taqman probe specific for the ZIP code in a singleplex setting.

Padlock probes are designed in such a way that one target site, the anchor, has a higher melting temperature (Tm) than the ligation temperature. The other target site has a Tm lower than the ligation temperature, the combination of which results in a synergistic, highly specific ligation reaction (Szemes *et al.*, 2005).

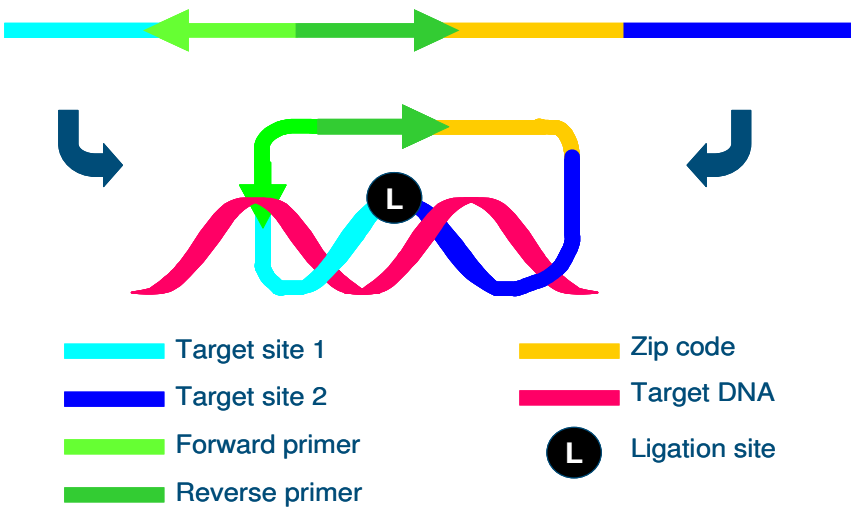

Figure 3. Scheme of padlock probe ligation reaction in the presence of target DNA.

Results

We have developed a microarray with 50 unique complementary ZIP-codes for the detection of padlock probes, spotted in quadruplicate. Specificity of these arrays has been tested with a pool of labelled complementary ZIP nucleotides as a proof of principle. Specificity was generally high, with a few exceptions which will not be used for future padlock probe development.

Initially, the ligation reaction was optimized with a single padlock probe with real-time PCR detection after the ligation step. Sensitivity was also tested in this way. In a dilution series of maize genomic DNA in herring sperm DNA, 0.1% was on the verge of reliable detection (Figure 4), similar to the sensitivity of conventional real time PCR (0.1% for most plant GM targets). These results warranted the development of more padlock probes and multiplex testing with microarray hybridization as final detection and identification method.

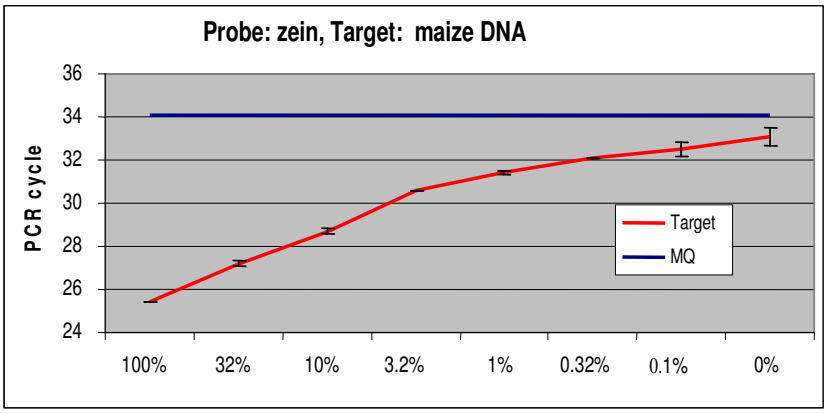

Figure 4. High sensitivity: Maize DNA diluted in herring sperm DNA to mimic different GMO percentages. Shown are the Ct values (PCR cycle at which a specific signal is detected) for various percentages of maize DNA and water (MQ) as a negative control. A correlation is shown between the amount of target and the appearance of signal. Signal from MQ is likely to be caused by primer-dimerisation and further work will also focus on eliminating these signals.

One of the main challenges for a multiplex system is the detection of targets with low abundance when other targets are up to a 1000-fold more abundant in the reaction mixture. In traditional multiplex PCRs highly abundant targets often inhibit the detection of low abundant ones. We therefore tested the ability of the padlock approach to overcome this in two sample types: a low abundant target in combination with one high abundant target (1000-fold and 100-fold); we used the IRMM standards for 0.1% and 1% Mon810 maize for this. The other type was one or two low abundant targets in combination with three high abundant ones (666- to 3-fold). For this we used either a wild type maize or a 50% Mon810 maize sample in combination with a mixture of wheat, oat and soy genomic DNA. All samples were analyzed with a mixture of five different padlock probes in the reaction mixture.

In the two-target samples both 0.1% and 1% Mon810 were detected. In the five- and four-target samples, 0.5% Mon810 and 1% maize were detected (Figure 5). The high abundant targets were detected in all cases. A positive signal was defined as all four spots having a signal to noise ratio of more than three. Based on visual inspection of the microarray scans, signals were scored as positive (+), more positive (++), or very positive (+++). The wheat specific probe showed aspecific positive signal when no wheat was present in the sample, though less intense than when wheat was present. No positive signals were observed for the 45 spots for which no probes were present. Also the Mon810, oat and soy specific probes were negative when their target DNA was not present in the DNA mixture (Table 1).

Table 1. Input and Output of the tested multiplex padlock reactions

input DNA(% w/w)					output (microarray signal)					
Mon810	Maize	Wheat	Oat	Soy	Mon810	Zein (maize spec.)	Wheat spec	Oat spec	Lectin (soy spec.)	neg. spots
0.1	100	-	-	-	+	+++	++	-	-	-

1.0	100	-	-	-	++	+++	++	-	-	-
0.05	0.1	33.3	33.3	33.3	-	-	+++	+++	++	-
0.5	1.0	33	33	33	+	+	+++	+++	++	-
5.0	10	30	30	30	++	++	+++	+++	++	-
-	0.1	33.3	33.3	33.3	-	-	+++	+++	++	-
-	1.0	33	33	33	-	+	+++	+++	+++	-
-	10	30	30	30	-	++	+++	+++	++	-

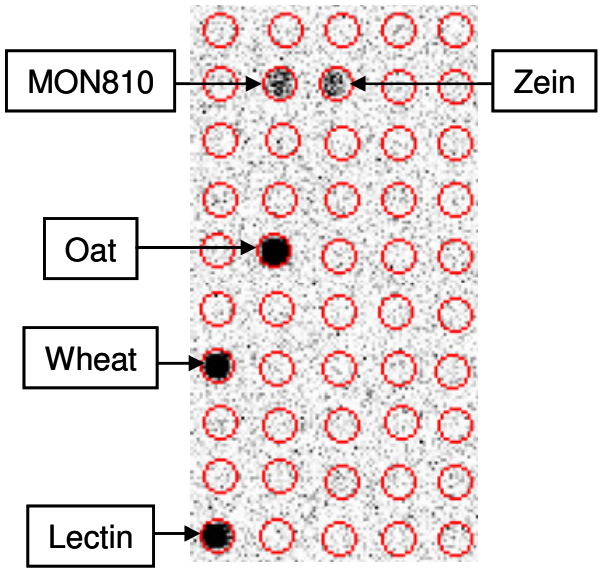

Figure 5. High sensitivity of the padlock ligation system in a five-target sample. Shown is a black and white image of one of the four replicates on a microarray slide. Specific spots are identified. This picture shows positive results for a DNA mix containing 0.5% MON810 maize, 1% wild-type maize and 33.3% of wheat, oat and soy DNA, analyzed with a five-probe reaction mixture.

Discussion, future plans and conclusions

RIKILT has shown the feasibility of padlock ligation in combination with universal PCR and microarray detection for sensitive GM detection in a complex mixture of plant DNA. High sensitivity (between 0.5 and 0.1%) was observed even when other positive targets were present up to a 1000 fold more. For now, the system has been tested in a five-plex setting.

Future experiments will have to reveal the level of multiplexing that can be reached with this technique. Probes for GTS 40-3-2 soy and TC1507 maize are currently under investigation. In the future, also element profiling will be an important aspect in padlock probe design. This will be important for detection of non-approved GM varieties, in which known elements must be used, because we presently cannot predict actual construct sequences. Non-approved varieties can then be detected when a combination of elements is observed that is not present in any of the approved varieties.

One theoretical advantage of microarray identification over identification based on specific product length is that a virtually endless amount of probes can be designed with similar length. This could lead to more equal PCR efficiencies, as the detection is not based on a specific length of the product, but on the presence of a specific DNA sequence (ZIP code) within the product.

3.1.7 SNPlex™ technology for GMO detection purposes (INRA, partner 1)

INRA-MDO partner is trying to adapt SNPlex and assess its possibility to detect unauthorised GMO in combination with a matrix approach. The SNPlex Genotyping System is based on the oligonucleotide ligation/PCR assay (OLA/PCR) with a ZipChuteTM probe detection (Tobler *et al.*, 2005). It is a high throughput SNP genotyping system, allowing the detection of 48 SNPs in one sample. As far as GMO analysis is concerned no system allowing detection of 48 targets in the same tube has yet been developed, and therefore SNPlex technology could be of interest. But in order to be validated and applied to GMO analysis, the new methods should fulfill certain performance criteria, have a detection limit matching the legal labelling threshold of 0.9% relative GMO concentration, and be cost effective.

Presentation of SNPlex technology

SNPlex is a sequential combination of Ligase Chain Reaction (LCR) and multiplex PCR with universal primers. It is a multistep (eight steps) procedure designed for automation. Ideally it requires two liquid handling robots and a plate wash station, while a pre-PCR working area is essential (Tobler *et al.*, 2005). For simplification purposes, it can be divided into three steps as shown in Figure 6.

The first step consists of the generation of genotype specific products through multiplex Oligonucleotide Ligation reaction (OLA). Three different oligonucleotides described in Figure 6 are mixed together, once the two oligonucleotides matching the sequence are hybridised and ligated, the second step is performed.

The second step is an amplification step with universal PCR primers. The newly amplified fragments being biotinylated are fixed on a streptavidin support and the unfixed probes are washed away.

The third step is the decoding step. The specific ZipChuteTM probe is hybridised to its complementary ZipCodeTM sequence. After washes to eliminate the unspecific unhybridised probes, the ZipChute fixed to the amplified sequences of interest are eluted and detected by capillary electrophoresis. The results are then analysed by GeneMapper software (Applied Biosystems).

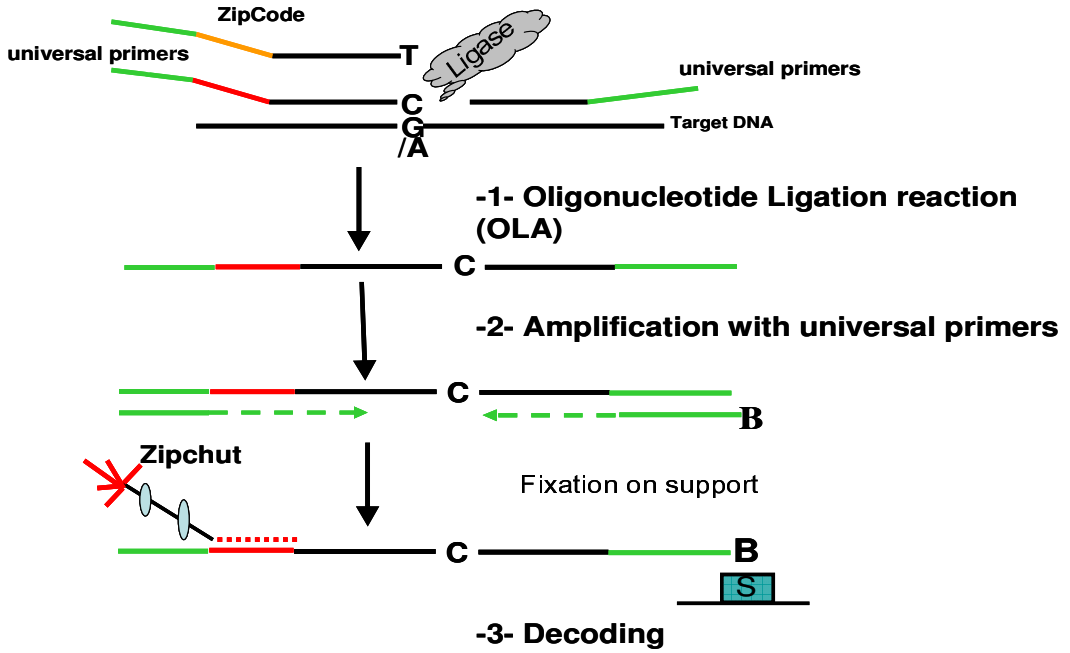

Figure 6. Schematic representation of SNPlex technology. It can be schematically divided in three steps 1.OLA reaction, 2.Amplification, 3.Decoding. Green strokes represent universal primers. Orange and red strokes are ZipCode sequences. These sequences are specific to

each oligonucleotide and complementary to ZipChute sequence (red doted strokes). Fluorescently labelled ZipChute probes are hybridized to complementary ZipCode sequences that are part of genotype specific amplicons.

The trick lies in the specific 3' nucleotide position in the 5' oligonucleotide, and the fact that only a perfectly matching nucleotide in this position will permit ligation of the two oligonucleotides, and consequently binding of the ZipChute sequence to a solid support for detection. Adaptation to GMO detection is therefore a question of designing the appropriate oligonucleotides and obtaining a satisfactory specificity and sensitivity.

Adaptation of SNPlex to GMO detection

In order to use SNPlex for unauthorised GMO detection with a matrix approach, a panel of 48 targets was carefully chosen and the first experiments were run to test the performance criteria of the method.

Design of the targets panel

To set up a panel of targets, GMO sequences were retrieved from databases, patent applications and validated tests. Among all these sequences, 48 targets were chosen and submitted to the SNPbrowserTM software (http://marketing.appliedbiosystems.com/mk/get/snpb_landing?isource=fr_E_RD_www_allsnps_com_snpbrowser) to check for compatibility of the sequences. A panel of 47 compatibles probes was finally ordered. It allows the identification of Bt10, Bt176, Mon863, T25, Bt11, CBH351, Mon810, TC1507 and detection of commonly used elements for GMO development, like promoters (P35S, Pact), terminators (T35S, Tnos) and genes of interest (*cry* genes, *cp4-epsps*, *bar*, *pat*). Eight plant species (maize, wheat, potato, tomato, rice, sugarbeet, rapeseed, cotton) as well as three donor organisms (CaMV, *Agrobacterium*, and *Bacillus thuringiensis)* are also detected with this panel. Thus a panel allowing for detection of several GMOs including unauthorised events has been established. However other panels could easily be designed for specific purposes (e.g. maize specific…).

Test of the performance criteria

SNPlex is a qualitative method, which with the use of the panel developed and a matrix approach should allow for multiplex identification or detection of GMO in a complex sample. For that purpose, it should fulfil some criteria like specificity and a low limit of detection (LOD). Experiments were performed on a selection of 192 DNA samples in duplicates extracted from GM or non GM-plants as well as Certified Reference Material (CRM). DNA samples from *Agrobacterium*, *Bacillus thuringiensis* and CaMV were also included in the test, as the panel contains probes for control of donor organisms. All samples could be analyzed showing that the panel can detect specifically 44 targets. Out of the 47 probes, one is a-specific and two others do not hybridise. This fact (three to four unspecific targets) is commonly seen when SNPlex is used for genotyping. Therefore the panel design for GMO analysis is as efficient as a panel for genotyping. All the reference gene probes could identify the corresponding species and no cross reaction was observed. The probes used for control of donor organisms were also specific. In one sample, as many as 11 targets were detected simultaneously. Analysis of the 384 samples was done in only three days and the technique offer the possibility of analysing up to 1536 samples (4 plates of 384 samples) at the same time. Results are reproducible and 40ng of DNA are sufficient for the analysis. However the LODrel as observed (10%) was rather high compared to the legal threshold of 0.9%. Notably, when multiple events where mixed at 10% relative concentration, all the events in the mix were detected.

Limitation of the SNPlex for GMO detection purposes

In the framework of developing new technologies for GMO analysis, a specific SNPlex oligonucleotide panel of 47 targets was developed and tested on DNA samples extracted from GM or non GM plant. To our knowledge it is the first time that so many GMO targets could be simultaneously screened for in the same tube. The time to run the experiment, excluding DNA extraction, is less than 3 days and 1536 samples can be analysed in one run. The specificity and the number of targets to be detected in one sample are the main advantage of the technique.

However, in our first tests the LOD is unacceptably high (10%) compared to the 0.9% labeling threshold legally required. Therefore the LOD still need to be improved and more experiments are being performed to try to fulfill this requirement. In case of food or feed analysis a high LOD could be a real bottleneck. However when seeds are concerned it is possible to sub-sample, and the use of sampling plans like sampling plan by attribute (Kobilinsky & Bertheau, 2005) will allow detection of a low amount of GMOs. Since SNPlex is a qualitative technique, producing simple presence or absence binary responses, this approach may be particularly fit for combination with sampling by attribute. As SNPlex is a high throuput technology, increasing the number of analyses by subsampling would be feasible.

The laboratories which should use this technology are enforcement laboratories. As it is a new technology none is equipped for such a methodology. The advantage of high throughput could thus be a problem because the SNPlex require automation and robots which are costly. So the transferability of the SNPlex technology may be a challenge.

The most promising aspect of SNPlex technology is the multiplexing potential, while cost-effectiveness and scope are still challenging in relation to GMO detection.

3.2 Signal amplification methods

3.2.1 Bioluminescent Assay for Real Time/ BART (Lumora)

The BART (bioluminescent assay for real-time) follows the accumulation of pyrophosphate (in a sample) that necessarily occurs as dNTPs are incorporated into amplicon. As such, the level of pyrophosphate indicates the extent of amplification and hence the presence or not (and quantity) of target nucleic acid in the sample.

The biochemistry of BART is an adaptation of the 'Enzymatic Luminometric Inorganic pyrophosphate Detection Assay, or "ELIDA" (Figure 7).

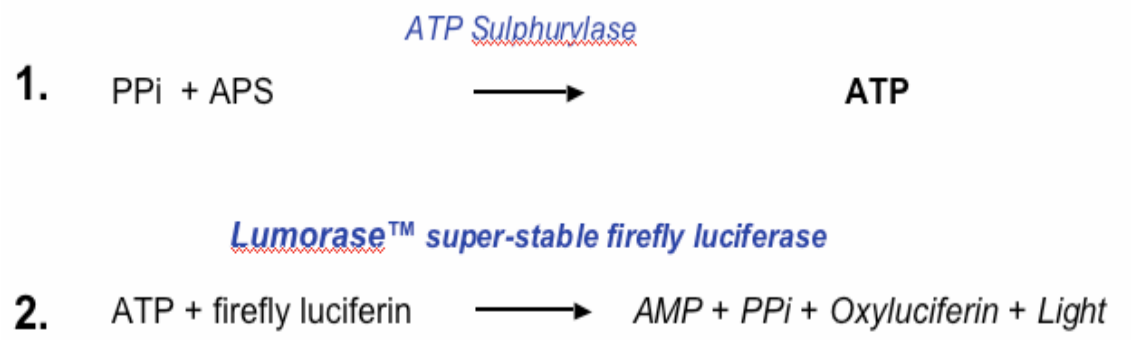

Figure 7. The two-enzyme coupled assay for pyrophosphate (PPi) detection: an adaptation of the 'ELIDA' assay (Nyren & Lundin, 1985)) that utilises highly thermostable firefly luciferases.

Unlike previous applications of the ELIDA assay (most notably Pyrosequencing), BART allows pyrophosphate levels to be assessed in real-time over long periods and harsh conditions (>55-60˚C for 2 hours or more). This allows real-time analysis of the extent of an isothermal amplification reaction.

A unique feature of BART outputs is that they are associated with both an increase in light intensity as amplicon is generated, and a subsequent rapid decrease in light. As such the outputs show a light peak in time; the time to reach this light peak is a function of the amount of target DNA in the sample at the beginning of the reaction (Figure 8).

Therefore the key features/benefits of the BART outputs are:
the key parameter: time to light peak, is not dependent on absolute light intensity, this greatly simplifies data interpretation and required hardware
the time to light peak has been shown to correlate with the amount of specific target template at the beginning of the reaction.
the measurements are made in real-time in a closed-tube format.

BART can not be used with conventional PCR or LCR as the luciferase enzymes are insufficiently stable to operate at 95˚C (and additionally LCR does not release significant amounts of pyrophosphate). However, BART gives excellent results with at least 5 different isothermal methods now available. In this project, we have utilised LAMP (loop mediated isothermal amplification- see Section 3.1.5).

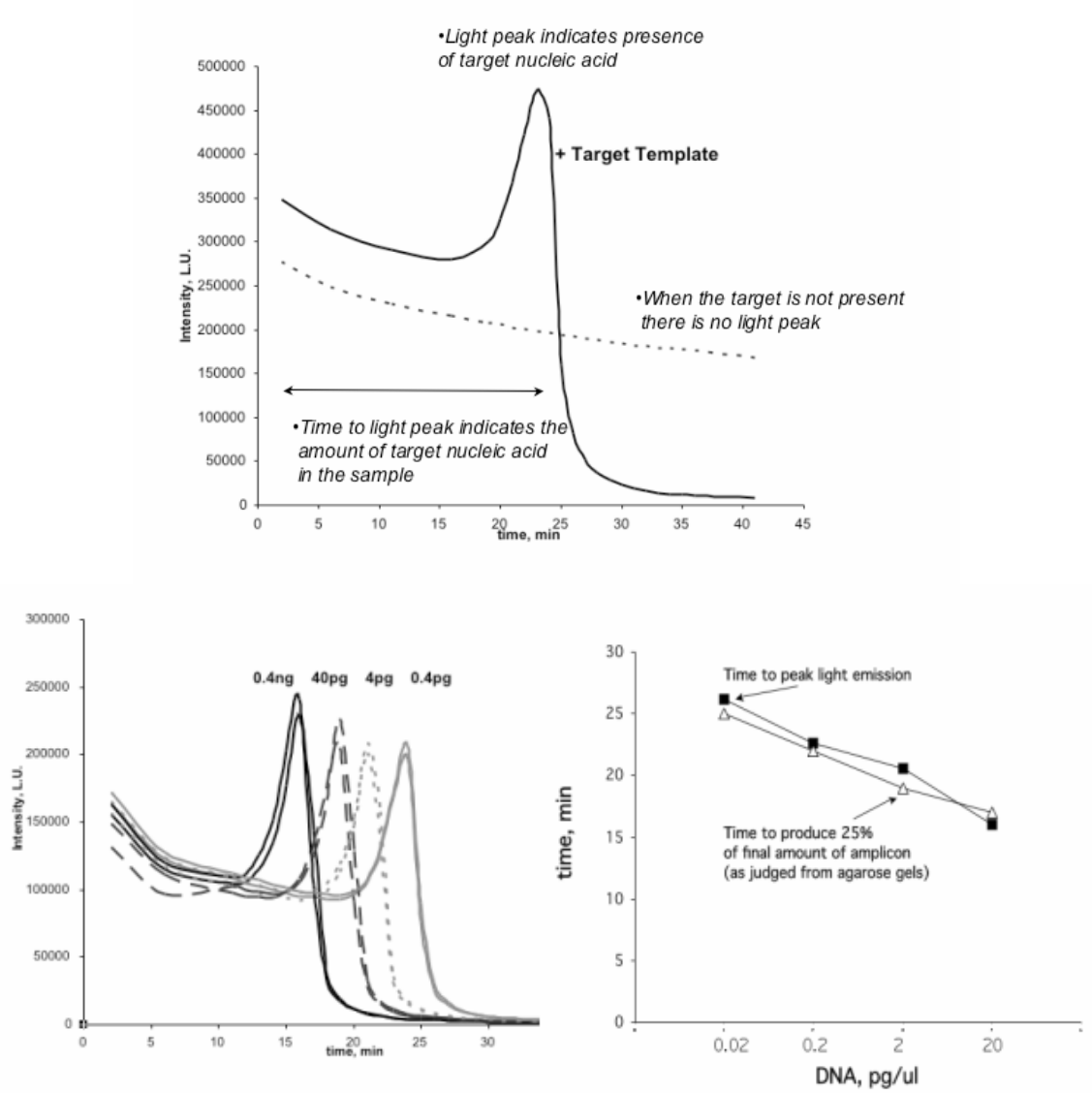

Figure 8. Upper) typical light profile of a positive and negative amplification reaction. In the positive sample, as PPi is released by generation of amplicon, it is converted into ATP causing an increase in light (the background light intensity is caused by dATP reacting with luciferase), however, as amplification progresses the light intensity drops to below the level of the control. This drop in light intensity is caused by a number of factors including inhibition of luciferase by high [PPi]; **Lower) the quantitative nature of BART is shown (using LAMP) using different template concentrations.**

Analysis was carried out using LAMP coupled with BART for event GTS 40-3-2 (Roundup Ready soybean, Monsanto) as well as for events RF3 and MS8 of Bayer Invigor canola. RF3 and MS8 correspond to the two parental constructs present in single copy (hemizygous state) of the Invigor hybrid canola grown commercially. Single copy sensitivity was obtained for both RF3 and MS8 primer sets (Figure 9), whereas the primer set for GTS 40-3-2 was found to require further optimisation and primer redesign for equivalent detection limit. We conclude that LAMP amplification coupled to BART can provide a sensitive detection of specific GMO events, and that BART provides a reliable and simple way of following DNA amplification in real-time. Due to the simplicity of the hardware requirements compared to real-time PCR (constant temperature for sample, output detection by CCD chip or camera), BART is highly suitable for developing very low cost apparatus capable of carrying out tests on dispersed sites to a low budget.

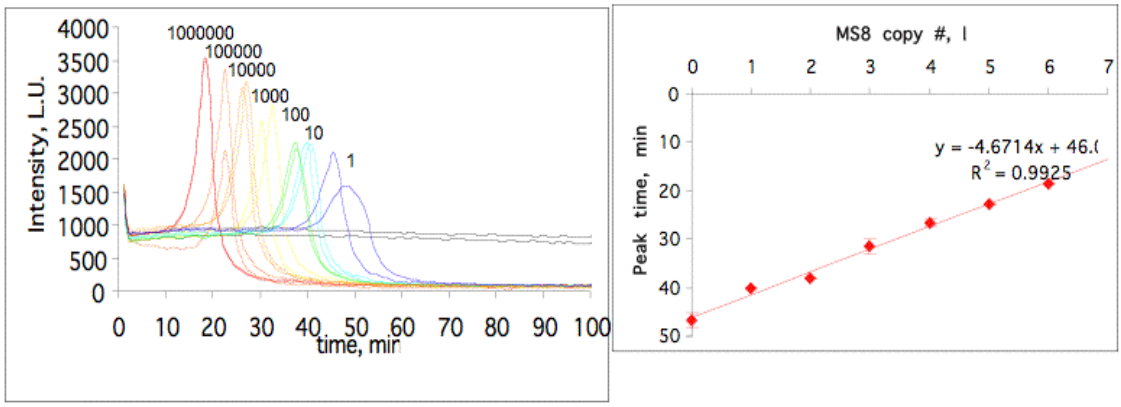

Figure 9. Example of detection of MS8 within 20-50 minutes, with sensitivity to single copy. Left: raw BART outputs showing light intensity against time. **Note the duplicate negative controls show a flat line with residual signal maintained.** Right: plot of log concentration against time to peak.

3.2.2 Real-Time Immuno-PCR/ RT-iPCR (BfR)

The BfR developed a new strategy for the detection of GMO specific DNA, using a combination of complementary hybridisation, immunological detection and signal enhancement by PCR, still avoiding individual event specific amplification reactions in parallel. The principle is based on Real-Time Immuno-PCR (RT-iPCR), a method that is successfully applied in protein research for the ultra-sensitive detection of proteins (Barletta, 2006; Niemeyer *et al.*, 2005).

The principle of the method is shown in Figure 10. In a first step, biotinylated target DNA binds complementarily to immobilised PNA (peptide nucleic acid) probes in microtiter cavities.

The application of DNA hybridisation probes for the capture of DNA molecules has several disadvantages. Sensitivity and selectivity are often insufficient. Oligomer DNA probes offer good selectivity, but only a low stability of the duplex formed with the target sequence. Using longer probes (e.g. PCR products) improves sensitivity, because the duplex stability is higher, but selectivity is reduced (Weiler *et al.*, 1997, Guo *et al.*, 1994). Furthermore, hybridisation of DNA probes and target DNA has to be carried out in the presence of high salt concentrations, which are needed to counteract the repulsion between the negatively charged DNA strands. Under those conditions, however, target DNA tends to form secondary and tertiary structures within the molecules. This impairs target sequence accessibility. In order to overcome these limitations, we decided to use PNA probes for hybridisation. PNAs are synthetic DNA analogues with a polypeptide, usually formed of N-(2-amonoethyl)-glycine units, in place of the sugar phosphate backbone. Their neutral charged backbone increases the binding strength to complementary DNA compared to the stability of the respective DNA duplex, which is of benefit, if stringent washing after hybridisation is to be applied. With PNA probes, mismatches have a more destabilising effect on duplex stability, thus improving discrimination, compared to the use of DNA probes. Due to their uncharged nature, PNAs also permit the hybridisation to DNA samples under very low or even no salt conditions, since no inter-strand repulsion (as occurs between two negatively charged DNA strands) needs to be counteracted. As a consequence, the target DNA has less secondary and tertiary structure and is more accessible to probe molecules. Due to the properties described above, PNA probes bind stronger and faster to complementary DNA than DNA probes. They combine the resulting higher sensitivity with higher specificity due to their discriminative binding features. Furthermore their resistance to nucleases and proteases makes them robust in handling. (Weiler *et al.*, 1997; Ray & Norden, 2000)

In a second step, after removal of unbound DNA by washing and after blocking of unspecific protein and DNA binding sites, preformed protein-DNA conjugates are added and bind to the hybridised biotinylated DNA by immunological interaction. The conjugates consist of an anti-biotin antibody and a double-stranded DNA molecule that serves as a universal *reporter-DNA* for all samples. Unbound conjugates are washed off. Bound conjugates are quantified by Real-Time PCR targeting a region of the reporter-DNA sequence which is not present in any GM construct or plant derived DNA. The same Real-Time PCR system is used for all microtiter wells. Positive signals are only considered if a cut-off threshold has been exceeded. The cut-off threshold is defined by the background intensity of negative control samples.

Preliminary results show that the RT-iPCR approach, only used for protein detection before, is principally applicable for the detection of DNA. The main focus of optimisation will be on the improvement of sensitivity, namely signal-to-noise ratio.

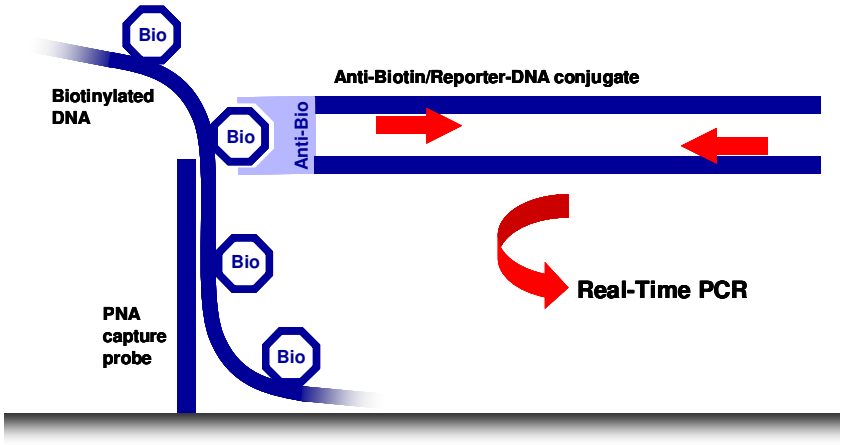

Figure 10. Principle of Real-Time Immuno-PCR for detection of DNA. Biotinylated DNA binds complementarily to immobilised PNA capture probes. Anti-biotin/reporter-DNA conjugates are added. Bound conjugates are detected by Real-Time PCR.

3.2.3 Multiplex capillary electrophoresis, SNaPshot labelling (Matforsk)

Snapshot labelling is a cyclic labelling method where probes binding to PCR products are labelled with fluorescent dideoxynucleotides. The labelled probes can thereafter be detected by capillary electrophoresis. Alternatively a PCR can be performed with fluorescent primers. These methods are much more sensitive and have a vastly higher resolution than for example agarose gel electrophoresis, and are therefore suitable for detection of weak signals. The methods must be used in conjunction with PCR to obtain the required sensitivity. We routinely use capillary electrophoresis for detection in both one step PCR, 2 step PCR and ligation mediated PCR, and routinely use SNAPshot for the 2 step PCR method. Regarding SNAPshot, the method as such is developed earlier, but there will always be some trial and error when selecting probes and conditions (some probes do not function properly or are labelled in the absence of template).

3.2.4 Primary and secondary phycoerythrin labelling of DNA for direct microarray hybridisation and detection (NVI)

Direct hybridisation and detection of genomic DNA on microarrays may potentially be exploited for detection of almost unlimited numbers of diverse sequence targets simultaneously, provided that the resulting signals from positive hybridisations are distinguishable from negative signals. Sensitivity is therefore a challenge. At the NVI we are exploring this approach as a tool to obtain some basic sequence information on inserted (foreign) DNA elements in single GM samples, as a means to rapidly characterise and further determine the potential risks associated with these elements. Such a tool is not primarily fit for routine application but may be urgently needed when there is strong suspicion of illegal release of a GMO and no details on the genetic content of the putative GMO is available.

The methods developed at the NVI require relatively large quantities of pure DNA (approximately 100 µg per array). The labelling system was established on the model plant Arabidopsis thaliana. Total genomic DNA was fragmented using DNase I and successively end labelled using terminal transferase and biotinylated dideoxynucleotides. After hybridization, the biotinylated DNA was labelled using streptavidin coupled with phycoerythrin. In addition, phycoerythrin-labelled anti-streptavidin antibodies were added to increase signal intensities. A modified protocol for doing this using standard reagents and our custom design microarrays was developed in-house. At the NVI we conclude that signal amplification using streptavidin-bound phycoerythrin in combination with anti-streptavidin antibodies coupled with phycoerythrin yields sufficient fluorescent signal for the use of total genomic DNA in combination with Affymetrix-type microarrays. A manuscript reporting on the experiments carried out with direct genomic DNA hybridisation to custum designed arrays is submitted. An additional manuscript is in preparation, reporting on rice. Additional experiments are in preparation and new custum designed arrays are presently being designed.

4 Summary of results, discussion and conclusion

4.1 Target amplification methods:

The NIB (Partner 6) has developed a target amplification procedure based on NASBA (Nucleic Acid Sequence-Based Amplification). After the generation of specific template DNA by use of bipartite primers, which contain sequences of RNA polymerase promoter elements, the target DNA can be amplified linearly by universal primers, enabling a multiplex set-up and subsequent detection of target sequences on arrays. The customized NASBA protocol resulted in linear and equal amplification of amplicons in up to triplex reactions. Expansion of multiplexing and improving of on-chip detection is planned.

Matforsk (Partner 29) is working on two target amplification approaches. MQDA-PCR (multiplex quantitative DNA array based PCR) is based on a 2-step PCR. After a few PCR cycles with bipartite primers, as yet targeting up to 8 different targets in the same reaction, the unused primers and/or polymerases are degraded. Universal primers are used for further amplification of the targets, after binding to the bipartite primers' "head" region. Detection can be done by hybridisation of labelled probes to complementary sequences on a DNA array or by capillary electrophoresis. Up to 8 different GMO targets were detected quantitatively with a sensitivity of at least 0,1% GMO with little interference between signals from different GMOs.

In addition to MQDA PCR, Matforsk uses the ligation mediated amplification system MLPA, which has been reported to work in a 40-plex system before. So far, Matforsk have developed a 5-plex MLPA procedure with capillary electrophoresis detection of amplicons for different GMO targets, intending to include more GMO events in the future.

The BfR (Partner 11) applies Multiple Displacement Amplification (MDA) for whole genome amplification of GMO template DNA, in order to provide large amounts of sample DNA for subsequent identification of GMO targets by hybridisation to specific probes in a microtiter format. An amplification of up to 17000-fold results in amplified DNA (mdaDNA) of high quality, representing the initial proportion of target sequences with minimal bias. NVI (Partner 7) reached similar conclusions and also tested a more targeted amplification by MDA, using sets of specific oligonucleotides targeting different GMO elements in the MDA reaction. However, this set-up did not lead to a significant amplification of the targeted regions.

Lumora (Partner 28) uses Loop-Mediated Amplification (LAMP) for isothermal amplification of target DNA. Dumbbell-like structures are formed and replicated by several specific primers, which recognize four different target sites instead of just two in PCR, leading to highly specific amplification. Lumora designed and tested LAMP primers for the 3' junction of the GTS 40-3-2 (Round-up Ready Soya) insert. Specific products were visualised by gel electrophoresis and using the BART assay (see below).

The RIKILT (Partner 9) has explored the padlock probe ligation approach for the multiplex-detection of GMOs. If a padlock probe hybridises specifically to a target sequence, ligation occurs and results in a circular molecule. Subsequently, all circularized padlock probes are amplified by universal PCR. Each padlock probe contains a unique ZIP code which can be detected on a microarray. Multiplexing with up to 1500 padlock probes in a single reaction has been done before. The RIKILT has shown the feasibility of the method for GMO detection in a 5-plex set-up with a sensitivity of 0,5-0,1% GMO even when other positive targets were present in up to 1000-fold higher relative concentrations. A microarray for 50 ZIP codes has been developed and the expansion of the multiplexing level is planned.

The INRA (Partner 1) adapted the SNPlex Genotyping System (Applied Biosystems) for the detection of GMOs in combination with a matrix approach. The system is based on a combination of multiplex Oligonucleotide Ligation Reaction (OLA) and universal PCR, followed by a ZipChuteTM probe detection. A panel for testing of 44 different GMO targets in the same

tube by the SNPlex technology was developed successfully for an LOD of 10% GMO. In one sample, 11 targets were detected at the same time.

4.2 Signal amplification methods

Lumora applies the BART assay (Bioluminescent Assay for Real-Time) for real-time analysis of the extent of amplification in their LAMP assay (see above). When dNTPs are incorporated into an amplicon, pyrophosphate is released as a by-product. Using luciferase, the accumulation of pyrophosphate is assessed by measuring the time to a light peak as a function of the initial amount of target DNA in the sample.

The BfR explores a new method for the detection of target DNA by combining a hybridisation step with immunological detection and a subsequent universal PCR. The principle is related to Real-Time Immuno-PCR, a protein detection method. After hybridisation to PNA capture probes, biotinylated target DNA is detected by addition of anti-biotin/reporter-DNA conjugates and subsequent quantification of bound reporter-DNA by Real-Time PCR. Feasibility of the method has principally been shown for the detection of short DNA targets. Optimisation will include testing of different GMO targets and improvement of signal-to-noise ratio.

Matforsk uses Snapshot labelling for detection of GMOs by capillary electrophoresis after 2-step PCR. Snapshot labelling is a cyclic labelling method for labelling of detection probes with fluorescent dideoxynucleotides.

The NVI uses streptavidin-phycoerythrin conjugates for the detection of biotinylated target DNA. Additionally, for further signal enhancement, phycoerythrin-labelled anti-streptavidin antibodies are applied.

Considering the prospectively growing challenge of monitoring and controlling the co-existence of GM and non GM crops, the identification of several GMOs in parallel is the central criterion for the ability of a method to substitute the PCR as a standard method for GMO analysis. Up to now, multiplex analysis in one-tube set-ups was achieved to varying degrees by the different approaches within the Co-extra project. Several partners plan to expand the level of multiplexing in the next phase of the project. RIKILT for instance has successfully established a 5-plex setup for their padlock probe ligation approach, potentially with a wide scope for higher multiplexing being left, as they report that this approach has been carried out in multiplex with up to 1500 probes in a single reaction for SNP detection before. INRA successfully established testing for 44 different GMO targets at the same time with a sensitivity of 10% GMO content, using SNPlex technology. Despite the drawback of a relatively high LOD, SNPlex being a high throughput technology might still allow the detection of low amounts of GMOs in seeds by the use of sub-sampling plans.

Cost effectiveness will be a critical point for the evaluation of the methods concerning their suitability for standard analysis. Some of the methods, e.g. INRA's SNPlex technology and NVI's high density microarrays, require complex technical devices, thus making adaptation for broad applications in standard GMO analysis difficult. Nevertheless, if the methods are reliable, then the capacity for automated high troughput screening might compensate for costly equipment, at least for some laboratories or for particular applications. The number of targets covered by a test (level of multiplexing) is another important factor.

Several of the approaches investigated are based on or offer the possibility of integration with arrays, e.g. NASBA (NIB) and MQDA-PCR (Matforsk). NIB is working in collaboration with EAT. EAT developed an array on which the targets amplified by the NASBA method will be hybridised. The micro-array bears capture probes complementary to the NASBA amplified target. Currently the micro-arrays are ready for tests and were sent to the NIB. The first results are expected within the next months. NVI have designed Affymetrix-type microarrays (Affymetrix, Inc.) for the purpose of detecting both unknown and well characterized genetic modifications in plants using a whole-genome based approach. The array design includes probes complementary to target sequences either from known GM constructs (selection markers, promoters, terminators etc.) or probes that have been specifically designed to detect allochthonous DNA sequence motifs within the context of well characterized plant genomes. In

order to screen complete genomes in an unbiased way using microarrays, methods for total (genomic) DNA preparation and labelling are also needed and have been established. Additionally, Multiple Displacement Amplification (MDA) can be used for the unbiased amplification of genomic DNA in order to provide large amounts of high quality sample DNA for array hybridisation.

An additional characteristic of the different approaches is their suitability for quantification. Quantitative signals were detected in 8-plex MQDA-PCRs (Matforsk), with a sensitivity of 0,1% GMO and little influence on specific GMO signals in presence of total GMO up to 7%. The method is currently being compared with results from single Real-Time PCR. Semi-quantitative results may be expected from linear target amplification by NASBA (NIB) and NVI Affimetrix-type microarrays. Padlock probe ligation (RIKILT) has not been tested for quantitative purposes yet. SNPlex (INRA) and Real-Time Immuno-PCR (BfR) allow qualitative detection solely.
Within the Co-Extra project, several approaches for target and signal amplification methods other than PCR have been explored by different project partners and promising results have been achieved so far. While several of the methods applied for GMO detection offer good prospects of further exploration and possible implementation in standard GMO analysis, none of the methods has yet proved to be sensitive, reliable and robust in routine applications. Future studies will focus on expansion of multiplexing, further optimisation of the methods and testing of their applicability for routine testing for GMOs.

5 References

- Barletta, J. (2006): Applications of real-time immuno-polymerase chain reaction (rt-IPCR) for the rapid diagnoses of viral antigens and pathologic proteins. Review. Molecular Aspects of Medicine 27: 224-253.
- BATS (2003). Genetically modified (GM) crops: molecular and regulatory details. http://www.bats.ch/gmo-watch/GVO-report140703.pdf. 199pp.
- Blanco, L., Bernad, A., Lazaro, J.M., Martin, G., Garmendia, C. & Salas, M. (1989): Highly Efficient DNA Synthesis by Phage phi29 DNA Polymerase. Journal of Biological Chemistry 264 (15): 8935-8940.
- Blanco, L. & Salas, M. (1996): Relating Structure to Function in phi29 DNA Polymerase. Journal of Biological Chemistry 71 (15): 8509-12.
- Dean, F.B., Hosono, S., Fang, L., Wu, X., Faruqi, A.F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., Driscoll, M., Song, W., Kingsmore, S.F., Egholm, M. & Lasken, R.S. (2002): Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci USA 99 (8): 5261-5266.
- Dunning, A.M., Talmud, P. & Humphries, S.E. (1988): Errors in Polymerase Chain Reaction. Nucleic Acids Research 16 (21): 10393.
- EC (2002). Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002
- EC (2003a). Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of 18 April 2003
- EC (2003b). Regulation (EC) No. 1830/2003 of the European Parliament and of the Council of 18 April 2003
- EC (2007). GM food & feed autorisation. http://ec.europa.eu/food/food/biotechnology/authorisation/index_en.htm
- Esteban, J.A.; Salas, M. & Blanco, L. (1993): Fidelity of phi29 DNA polymerase. Journal of Biological Chemistry 268 (4): 2719-2726.
- Eurobarometer (2005). Europeans and biotechnology in 2005: Patterns and trends. Eurobarometer 64.3. http://www.ec.europa.eu/research/press/2006/pdf/pr1906_eb_64_3_final_report-may2006_en.pdf
- Fukuta S., Mizukami Y., Ishida A., Ueda J., Hasegawa M., Hayashi I., Hashimoto M. & Kanbe M. (2004). Real-time loop-mediated Isothermal amplification for the CaMV-35S promoter as a screening method for genetically modified organisms. European Food Research and Technology 218: 496-500.
- Guo, Z., Guilfoyle, A., Thiel, A.J., Wang, R. & Smith, L.M. (1994). Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. Nucleic Acid Research 22 (24): 5456-5465.
- Hardenbol, P., Baner, J., Jain, M., Nilsson, M., Namsaraev, E.A., Karlin-Neumann, G.A., Fakhrai-Rad, H., Ronaghi, M., Willis, T.D., Landegren, U. & Davis, R.W. (2003). Multiplexed genotyping with sequence-tagged molecular inversion probes. Nature Biotechnol. 21(6):673-8. Epub 2003 May 5.
- Hosono, S., Faruqi, A.F., Dean, F.B., Du, Y., Sun, Z., Wu, X., Du, J., Kingsmore, S.F., Egholm, M. & Lasken, R.S. (2003): Unbiased whole-genome amplification directly from clinical samples. Genome Research 13 (5): 954-964.
- ISO, 2005. Foodstuffs – methods of analysis for the detection of genetically modified organisms and derived products – quantitative nucleic acid based methods. International Organization for Standardization. ISO 21570:2005.

- James C. (2006). Global status of commercialized biotech/GM crops: 2006. ISAAA briefs No. 35.
- Kobilinsky, A. & Bertheau, Y. (2005). Minimum cost acceptance sampling plans for grain control, with application to GMO detection. Chemometrics and Intelligent Laboratory Systems 75: 189-200.
- Luthra, R. & Medeiros, L.J. (2004): Isothermal Multiple Displacement Amplification: A Highly Reliable Approach for Generating Unlimited High Molecular Weight Genomic DNA from Clinical Specimens. Journal of Molecular Diagnostic, 6 (3): 236-242.
- Monsanto (2004). Monsanto Biotechnology Regulatory Sciences: A Recommended Procedure for Real-Time Quantitative TaqMan PCR for YieldGard Rootworm Corn MON 863, 2004; gmo-crl.jrc.it/detectionmethods/MON-Art-47-pcrMON863maize.pdf; 19.01.2005
- Mori Y., Nagamine K., Tomita N. & Notomi T. (2001). Detection of Loop-Mediated Isothermal Amplification Reaction by Turbidity Derived from Magnesium Pyrophosphate Formation. Biochemical and Biophysical Research Communications 289: 150-154.
- Nagamine K, Hase T, Notomi T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16:223-9.
- Niemeyer, C.M., Adler, M. & Wacker, R. (2005): Immuno-PCR: high sensitivity detection of proteins by nucleic acid amplification. Review. Trends in Biotechnology 23 (4): 208-216.
- Notomi T., Okayama H., Masubuchi H., Yonekawa T., Watanabe K., Amino N. & Hase T. (2000). Loop-Mediated isothermal amplification of DNA. Nucleic Acids Research 28 (12): e63
- Nyren & Lundin (1985): Enzymatic method for continuous monitoring of inorganic pyrophosphate synthesis. Anal Biochem 151:504-509
- Pinard, R., de Winter, A., Sarkis, G., Gerstein, M., Tartaro, K., Plant, R., Egholm, M, Rothberg, J. & Leamon, J. (2006): Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing. BMC Genomics 7 (1): 216.
- Ray, A. & Norden, B. (2000): Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. FASEB J 14 (9): 1041-1060.
- Ronning, S.B., Vaitilingom, M., Berdal, K.G. & Holst-Jensen, A. (2003): Event specific real-time quantitative PCR for genetically modified Bt11 maize (Zea mays). Eur Food Res Technol, 216: 347-354.
- Szemes, M., Bonants, P., de Weerdt, M., Baner, J., Landegren, U. & Schoen, C.D. (2005). Diagnostic application of padlock probes-multiplex detection of plant pathogens using universal microarrays. Nucleic Acids Res. 33(8):e70.
- Tobler, A.R., Short, S., Andersen, M.R., Paner, T.M., Briggs, J.C., Lambert, S.M., Wu, P.P., Wang, Y., Spoonde, A.Y., Koehler, R.T. Peyret, N.s, Chen, C., Broomer, A.J., Ridzon, D.A., Zhou, H., Hoo, B.S., Hayashibara, K.C., Leong, L.N., Ma, C.N., Rosenblum, B.B., Day, J.h P., Ziegle, J.S., De La Vega, F.M., Rhodes, M.D., Hennessy, K.M.& and Wenz, H.M. (2005). The SNPlexTM Genotyping System: A Flexible and Scalable Platform for SNP Genotyping. Journal of Biomolecular Techniques 16: 398–404.
- Weiler, J., Gausepohl, H., Hauser, N., Jensen, O.N. & Hoheisel, J.D. (1997): Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays. Nucleic Acids Research 25 (14): 2792–2799.