OSTC intermediate scientific report – Contract n°CP/42/322

Tracing and authentication of GMOs and derived products in the food-processing area

Coordinator:
Dr William Moens[1]
[1]Service of Biosafety and Biotechnology
Laboratory of Genetics and Traceability
Scientific Institute of Public Health,
Ministry of Social Affairs, Public Health and Environment
Rue Juliette Wytsmanstraat, 14
B-1050 Brussels BELGIUM

Promoters:
1. Dr William Moens: Institute of Public Health (ISSP)[1]
2. Dr Marc Deloose: Centrum voor Landbouwkundig Onderzoek (CLO)[2]
3. Pr José Remacle: Facultés Universitaires Notre Dame de la Paix (FUNDP)[3]
4. Dr Alfons Callebaut: Veterinary and Agrochemical Research Centre (VAR)[4]
5. Dr Gilbert Berben : Centre de Recherches Agronomiques(CRA)[5]

Scientific collaborators : Degand Ingrid[4], Deglas Valérie[3], Derwael Tinne[1], Gauthier Gilles[3], Janssen Eric[5], Mbongolo Mbella Guillaume[1], Merckaert Cindy[2] , Mylle Evelien[1], Dr Penninckx Iris[1], Taverniers Isabel[2].

[2]Centrum voor Landbouwkundig Onderzoek, Departement Plantenveredeling en genetica, Caritasstraat 21, 9090 Melle, Belgium.
[3]Unité de Biologie et Biochimie Cellulaire, Facultés Universitaires Notre-Dame de la Paix, rue de Bruxelles 61, 5000 Namur, Belgium.
[4]Department Quality and Safety, Veterinary and Agrochemical Research Centre , Leuvensesteenweg 17, 3080 Tervuren, Belgium.
[5]Centre de Recherches Agronomiques, Département Qualité des Productions Agricoles, Laboratoire de biologie moléculaire-détection OGM, Chaussée de Namur 24, 5030 Gembloux, Belgium

2. Introduction.

2.1 Context and summary.

Genetically modified organisms (GMO) are living organisms with engineered genetic modification of their genome. Most present GMOs bear an additional gene construct integrated in their genome after transformation. The additional exogenous gene(s) express a protein responsible for new agronomic trait of economical interest. .

In the European Union (EU), the social acceptance of GMOs entering the food/feed chains is poor whereas those intended for health or vaccinal uses are well accepted.
The Member states implemented – and are still implementing – new regulatory norms and rules to build up the predictability of the EU GMO market (Traceability) consumers confidence (labelling and threshold rules), protection of biological agriculture (co-existence) and operators' responsibility (liability).

In such a context, building a strong scientific and technological basement for the detection, identification and quantification of GMOs is part the tasks of the member states who are those fully responsible for regulations enforcement.

2.2 Objectives.

The main objective of the current project is to provide Belgium with some up to date technologies for detection, identification and quantification of GM crops and derived products noteworthy in food/feedstuff. It is also important for our state to participate to international scientific and normalisation efforts about analytical tools for GMO experimental traceability.
As anyone wondering to develop an analytical method, four classical questions should be answered:

- Which analyte should be detected?
- How to prepare the analyte?
- Is there reference material available?
- What are the most suitable methods to develop (cost/efficiency)?

2.2.1. Choice of the analyte

The analytical target – or analyte – is the GMO genomic sequence bearing the genetic modification that is specific for the GMO under analysis. The analyte can be directly targeted by Polymerase chain reaction (PCR)-based methods that allow amplifying a selected region of a genome from homologous oligonucleotides specifically bordering the selected analytical region. The amplified DNA fragment is then revealed, or quantified with classical means.
The genetic functionality of the genetic modification can be further traced by enzyme linked immunosorbent assay (ELISA) based methods at the level of the expressed recombinant protein.
In the present project, the selected analytes are targeted by internationally agreed PCR amplimers or by home-designed amplimers.

2.2.2. Preparation of the analyte

Preparation of the analyte from samples is a critical step of the analysis. Protocols are designed to cope with alterations of sensitivity and specificity. However, many reports show that some factors influencing the accuracy of quantification methods are still poorly mastered. Some paradigmatic, non-analytically based assumptions are currently describing the quality of a purified DNA in terns of size in terms of functionality if qualitative PCR. The so-called “matrix effect” is a used concept to explain (without in fact explaining) why there are still discrepancies in intra or inter laboratory exercises.
Therefore, the present project includes proposals to address the problem of the unavailability of “chemical descriptors” of DNA quality to be elaborated within the frame of Real Time PCR.
For this purpose, the composition of DNA extracts prepared by several DNA extraction methods are analysed by chromatographic separation of DNA and others constituents (promoter #4). Standards of DNA quality could be developed interactively versus their operability if RT-PCR. An extensive quantitative analysis of interference with PCR kinetics taking highly purified markers cloned into plasmids as reference. It is hoped that the resulting knowledge should allow discriminating between workable extracts from non-liable samples through the use of these analytical descriptors. Resulting purified DNA were then assessed for amplifiability by real time PCR (promoter #5).

2.2.3 Reference material

The development of reference material is usually a matter of scientific accuracy, quality management and traceability. Reference samples, controls, certified references are the basement of any analytical strategies. It is obviously the same with GMOs as target of analytical traceability.

In the case of GMOs, the simple availability of (non-certified) reference material is a major problem because it is primarily a political and regulatory issue.
In the practice, the companies are extremely reluctant to provide any significant amount of GM-crop material or DNA derived from it outside very narrow and difficult to conclude material transfer agreements.
Consequently, from the authority viewpoint, the whole enforcement and traceability dynamics of the EU market surveillance by the member states is dependent on the availability problem.
Secondly, when available, it is never in due time. In other words, should a market problem arise in the EU similar to the Starlink maize case in the USA, no single state would be able to control the market at time of real need.
Third, the companies themselves, while owners of the authorised Elite events, are usually not the producers of the seeds/grains at the source of IRMM powders as an example. Therefore availability is the first problem of the authorities, the companies and the screening/enforcement laboratories.

Presently, IRMM-Geel can provide certified reference material for four GMOs and two of them have availability and accuracy problems making them non-available in the practice. This corresponds to four of the GMOs commercialised in the EU between 1996 and 1997. However, there are 7 GMOs authorised on the EU market, and about 38 in the regulatory EU pipeline. Beside these 45 GMOs (7+38), more

than 500 gene constructs are under development in the non EU world and about 50 events are listed as “authorised” or even “deregulated” on the USDA web site.

As rough conclusion, non-availability in due time associated to exponential diversification are the three parameters of the problem of GMO reference material.
Our OSTC project aims noteworthy at addressing normative solutions to this problem under task A.1 - theme 1.

Traceability strategies and regulatory compliance

Present EU regulations applied to GMO related products tend to sectorialize with reference to the horizontal framework of directive 2001/18. They also tend to solve specific traceability issues either under the classical and cheap administrative traceability or under normative components such as thresholds of adventitious contamination (food, feed), de minimis threshold of contamination of biological agriculture, species-specific thresholds of impurities (seeds), threshold of non authorised GMOs, threshold for “GMO-free” products.
The assessment of these thresholds is part of the enforcement policies of member states. Practically, the enforcement laboratories must first screen samples collected by the authority, rapidly and at low cost. This where qualitative reference material are needed. These don’t need to be certified because they are used for qualitative detection of GMO and related crops in matrices. The average limit of detection of qualitative detection is currently around 25-60 copies of the analyte, in this case the GMO marker.
If non ambiguous positive matrices are identified by the screening step, then one can further address either the diversity of GMOs i.e. of GMO markers in a matrix as an “identification” step, or quantitate the abundance of the GMO marker per unit (ingredient, seed, etc…) as a “quantification” step. In these two cases, other types of markers and of reference material are required on a case by case. However the identification step could be coupled to the first screening step. This is an option developed by task A.

The idea is simply to make the GMO-specific genetic markers available in the public domain under the form of plasmid containing one marker as an insert.

We have proposed to develop plasmid-cloned analyte as reference material (task A.1 under theme 1 of the project). Plasmids are classic qualitative reference material in diagnostic kits of all types. Plasmids are easily available and can be produced quickly in large amount.
The plasmids proposed to be build under OSTC framework should contain event-specific junction markers (promoter #2), event-specific and or construct specific markers (promoter #1), and endogenous species-specific (promoter #1 and #2). These plasmids can also contain screening markers as promoter or terminator sequences. The different plasmids should be freely available from an official depository bank as the Belgian Coordinated Collection of Microorganisms (BCCM) (task A.2 under theme 1) for all members of the European Network GMOs Laboratories (ENGL).

Further, these plasmids overcome the issue of the variability of plant ploïdy and could be used as potential calibrators for the real time PCR (promoter #2) that is used to determine the amount of potential GMO food contaminants.

2.2.4 Analytical methods

Regarding the increasing diversity of GM plants arising on the world market, the "ideal" analytical tool should perform in a one shot experiment the screening of all GM plant markers. This technique of choice does not exist but we are taking advantage of a patented Belgian technology of one partner (promoter #3) that could allow a mass screening of agro-food products in a fast, cheap and reliable manner: The DNA biochips technology **(figure 1 in annex II under 6.2)**. The principle of this technique relies on the specific interaction of a PCR product to a single strand DNA (so-called capture probe) covalently linked on a solid surface. The PCR product results from an amplification of a DNA consensus sequence to several genetically modified events while the capture probe should be specific to a single event. It is then possible to screen one sample for different GM plant while reducing the number of PCR reactions, thus reducing the cost of an analysis.
This technology already exists for gene expression studies and screening of point mutation in gene fragment. The adaptation of the technology to the GM plant market is a major goal for the project.

2.3 Expected outcomes.

From the work performed by the different partners, we wondered to develop an efficient qualitative biochips that should allow to reduce the number of PCR enabling the authentication of a trace of GMO in food /feedstuff. Further, we wondered to get an available plasmid database with the different GM plant markers existing on the world marker to be able to improve the GMO chips and also to precisely quantify by real time PCR the trace of GMO contaminants in food. Altogether, this should guarantee the EU enforcement authority to be able to monitor efficiently GM plant and to restore the EU consumer confidence for the food/feed chain management.

3.detailed description of the scientific methodology.

Theme 1: Development of a bank of cloned genetic analytes as qualitative reference material for GMO detection and identification.

This goal has to be split along two axes:
Horizontally, the methodology applies:
- First to the selection of genetic markers as analytes, a selection that occur at the level of the regulatory-based, literature-based or patent-based documentation about the molecular characterisation of GMOs as or in products.
- Follows the laboratory steps of PCR primers design for qualitative of quantitative PCR able to target specifically the analyte under routine PCR format.
- Once this step is successful, the next steps are rather classical and essentially Maniatis-founded. The selected amplified genetic marker is cloned into a public domain plasmid or through a two step cloning procedure leading to identify a plasmid having three characteristics:
    - The core plasmid is public domain and internationally available. The selected plasmid is stable, growing in non recombinogenic bacteria
    - the plasmid itself contains an amplifiable analyte using the oligonucleotides defined in the method,
    - the insert has the expected size and the exact genomic sequence of the selected marker
- To the availability of an internationally recognised standard cloning procedure for the partners of the building of the bank. This is a first intersection with the vertical organisation of the project.
- To the availability of a standard format for the scientific documentation of the plasmid. This is the second intersection with vertical aspects.
- To the deposit of the plasmids together with the previously mentioned report in a single master Culture Collection under Budapest treaty, practically in Belgium in the BCCM culture collection of Gent.

Vertically, a single small laboratory cannot alone fulfil the need to build very rapidly the said bank in order to meet the three parameters of the availability problem.

In the course of this first year, we had two meetings with a committee of users that includes the JRC IRMM and the JRC IHCP. Scientists from these two institutes contribute to the development of the bank. Three additional non-financed partners have joined the initiative, INRA, DGCCRF, and BGVV.
All in all, there are about 80 analytes in the pipeline of cloning. This includes 26 analyte for theme 1 of this project. A web site is already available to get access to the list of plasmid. A first version was made available in 2002. However, for intellectual property rights reasons, and for strategic reason, it has been decided to build another web system as results of the new context explained in the next paragraph.

The project of the bank of cloned analytes was described at the ILSI meeting of December 2001, at two plenary meetings of the European Network of GMO Laboratories. On December 4th, ENGL was officially instituted by Commissaire Busquin and gathers 45 institution from the 15 member states. On December 5th, at

the first official plenary of ENGL, our plasmid project was approved unanimously by the panel of representatives making it a priority.
It was decided to recognise the bank as a private ENGL bank whose plasmids would be accessible to ENGL members for regulatory purposes. IRMM declared herself a typical user of the bank on that condition. The BCCM collection “LMBP” of Gent was selected as the unique master culture deposit for the plasmids.

Recently, companies have expressed their interest for the project of a bank of cloned analytes and are discussing the possibility to join the movement with the event-specific analytes of all their GMOs.

This project under theme 1 become more and more important with time and might require better co-ordination with BCCM, OSTC, ENGL and perhaps the 6-FP.

Theme 2: Qualitative GMO chips

The goal of this theme is simple: there are 7 GMOs on the EU market and 38 are soon to be assessed under the new directive 2001/18. The present qualitative screening methods need other methodological approaches. The GMO chips is a middle sized solution to cope with GMO diversity and the increasing lack of specificity of the so called “universal analytes such as 35S promoter and NOS terminator.

The first objective of this project was to determine consensus primers that able to amplify the elements common to different GMO but nesting single analyte specific for GMOs. Until now, the work focused on the 6 available GMO (Bt11, Bt176, Mon810, GA21, T25 and GTS-40-3-2). Three primer pairs have been selected from three different genes often presents in the GMO: PAT, EPSPS and CryAb genes. Table 1 in annex II under 6.2 summarises the name of the different primers set used, the amplified genes and the GMOs from which amplicon were amplified.

Theme 3: Analytical control for GMO quantification

Theme 3, taskC.2.1 and C.2.2: Influence of DNA purity on real time PCR

Choice of the matrix and grinding procedure

Analyses will be performed on soybean flour, taking advantage of our experience with this matrix (1). However, plasmid DNA purified on cesium chloride (2) could be used as reference material. The pLec2 plasmid (figure1 in Annex II under 6.2) which contains a specific insert from the soybean genome was then used as model to determine the amplifiability of the soybean genomic DNA.
The raw material was grind to get the finest powder to maximise the contact surface during the cellular lysis, thus increasing the efficiency of the DNA extraction. A first grinding step (grinder Retsh, model ZM100) allows obtaining particle less than 2mm in size. These particles were then brought to a size less than 500 □m by a second grinding step (this particle size fit with the one of the AFNOR norm concerning the GMO detection (3)).

Extraction of DNA

Five different methods were used to get DNA with "potential" chemical differences, namely:

- CTAB method (CTAB)
- Phenol/chloroform method (PC)
- "High pure PCR template purification kit" (HP) method from Roche Applied Systems
- Association of the semi-automatic device "Kingfisher™ " From ThermoLabsystems and the "Genomic DNA purification kit for Kingfisher" (TLS).
- Association of the semi-automatic device "Kingfisher™ " From ThermoLabsystems and the "Wizard® Magnetic DNA Purification System for Food" from Promega (PFF)

The two first methods are based on fractionated precipitation on using organic solvent. The other methods rely on the adsorption of DNA on silicate particles.
For two of these extraction methods (CTAB and PFF), RNase digestion is performed for RNA degradation. In other to compare effect of RNA molecules in DNA extract, the PC and the HP extracts were also treated with RNase (this digestion is usually not performed for these last procedures).
PC and CTAB extraction protocols are those mentioned in the pre-normative CEN/TC 275/WG 11 N148 document (4) with minor modification (K_2EDTA is replaced by Na_2EDTA). The protocols for HP, PFF and TSL extraction are performed in accordance with the kit manuals (5, 6,7)

Theme 3, taskC.2.3: Analytical description of DNA quality

DNA purification

HPLC seemed the most suited way to purify genomic DNA samples and to collect data on the impurities. Separation of short nucleic acids such as PCR amplification products, restriction fragments or oligonucleotides has been abundantly described in literature. The most used technique for these applications is anion exchange chromatography (AEC), as nucleic acids are negatively charged over a wide pH range. Although purification of genomic DNA by HPLC is poorly described, if described at all, AEC seems to offer the best perspectives for successful separation. Other purification options such as reversed-phase chromatography or size-exclusion chromatography were not investigated yet.
A photo-diode-array (PDA) detection system allows for the accumulation of spectral data in the UV and visible light of all components eluting from the column.

Choice of DNA matrix and DNA extraction methods (in collaboration with promoter #5)

Transgenic soybean has been well described and soymeal was therefore chosen as model matrix to carry out DNA extractions. Four different DNA extraction methods were selected. Two of them are commercial kits: High Pure PCR Template preparation kit from Roche (HP-extract) and the *Wizard® Magnetic DNA Purification System for Food* from Promega or genomic DNA kit from Thermolifesciences in combination with the King Fisher robot (KF-extract). The two other extraction protocols are phenol-chloroform (PC)- and CTAB-mediated extractions. Only CTAB

and KF-Promega extraction protocols include an RNase digestion step. All extractions, carried out on non-transgenic material, are described in the scientific report of promoter #5. Besides genomic soy DNA extracts, we have also tested a CsCl-purified plasmid containing a fragment of the LE1 lectin gene (pLec). We also tested different DNA standards or markers such as sheared salmon sperm DNA (fragments < 2000 bp, Invitrogen), phage digested DNA with HindIII (Invitrogen), unsheared genomic DNA (Roche) and high molecular weight markers (Invitrogen).

Choice of anion exchange chromatography columns

Most AEC columns have been optimised for short-chain nucleic acids, only. Separation of genomic DNA from residual components was carried out on four different AEC columns to select the most appropriated separating conditions. These are the DEAE 15HR, DEAE 8HR and the Gen Pak Fax columns from Waters and the DNA Pac PA100 column from Dionex. We have classified these columns as a function of their resolution and they were tested in that order. Different columns and conditions tested are described in appendix 1 in annex IV under 6.2.

Optimisation of qualitative PCR in salt gradients

Absorbance of eluting molecules is measured at 260 nm, that is the optimal wavelength for nucleic acid detection. Fractions are automatically collected and tested for the presence of the lectin gene (LE1) by classic PCR analysis.
PCR reactions were optimised by testing amplificability of the lectin gene in different reaction conditions (reaction volumes, sample volumes, cycle length). Briefly, 1 □l of DNA was amplified in a 30□l final reaction mixture containing 20mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mMdNTPu (dUTP used in place of dTTP), 300nM of each primers and 0.05U/□l of Platinium Tap DNA polymerase (PCR cycles and primers sequences are described in appendix 2 in annex IV under 6.2).

4. Detailed description of the intermediary results, preliminary conclusions and recommendations

Theme 1: Production and deposit of plasmid reference material.

a- Production of plasmids

All results concerning this task are in the **annex I under 6.2**.
A total of 38 plasmids (promoter #1 and #2) have been constructed for this first year of project. Promoter #1 mainly performed GMO construct-specific and species specific plasmids (table 1). Promoter #2 has constructed event–specific plasmids (table 2) as well as species-specific plasmids (table 3). Some of these GMO genetic markers are already in the final pUC18 vector (table 1, 2, 3) that is the deposit plasmid to the Belgian co-ordinated Collection of Micro-organism. Having such plasmid enables two main goals:

- Making easier the availability of GMO genetic markers as controls for experiments.
- Having controls with a known copy number of the genetic markers. This should facilitate a precise quantification of GMO trace in analysed sample.

In collaboration with the CLO (promoter #2) and a member of the Users Committee (IRMM Geel), a scientific dossier **(annex I)** has been standardised to harmonise the cloning procedures. Other non-financed partners have joined the collaboration WIV-CLO: The CRA, the INRA, the Institute of Health and Consumer Protection (Ispra), the IRMM (Geel), the DGCCRF (Strasbourg). The project of building a European bank of GMOs markers has been approved along the first plenary meeting of the European Network of GMOs Laboratories (ENGL) under the coordination of WIV-ISSP. The 45 ENGL members have expressed their interest and supported the project, most of them as “users”.

Moreover, it was agreed that the plasmids would be encoded as “pENGL” plasmids, deposited in the Belgian Co-ordinated Collection of Microorganisms (BCCM) of Gent as central master depository under the statute of full private deposit. The lawyers of the various concerned instances have estimated that this was the most secure type of deposit to prevent deviations in the public domain status and in the non-intended uses of the plasmids.

b- Quantitative PCR using plasmid (promoter #2)

Of each cloned plasmid, the DNA concentration was determined by spectrometry and the number of copies calculated from it. The concentration of cloned fragment is expressed as absolute copy numbers in order to be able to perform absolute quantification.
Specific primers were designed for each obtained first clone, amplifying a small fragment (100-200 bp). This primer pair was first tested qualitatively on a plasmid standard series. A fluorescent labelled TaqMan probe was then designed and used in combination with the specific primer pair, on a real-time ABI 7900 HT Sequence Detection System. Real-time PCR assays were optimised for primer concentration and probe concentration and linear calibration curves developed with the required high quality performance (regression coefficient of at least 0.99; slope of –3.3). Afterwards, if possible, certified reference materials from Fluka (IRMM-Geel) with precisely known content of GMO (expressed as a percentage), were prepared and

used as unknowns in real-time PCR reactions for relative quantification by means of the plasmid calibrators. The latter was done for RoundupReady soybean. One of the junction plasmid calibrator series (p-35S border respectively t-NOS border) quantified the number of copies of the event-specific junctions, while the *lectin* plasmid calibrator series quantified the number of copies of the *lectin* gene. By making the ratio and multiplying this number by 100, a percentage of RRS is obtained.
Primers and a TaqMan probe have been developed for 7 of the 9 junctions cloned and for the other 3 cloned fragments (not shown). Relative quantification through double absolute quantification has by now only been possible for RoundupReady soybean.

Altogether, we do demonstrate here the possibility to use plasmid DNA to gain accurate PCR quantification of GMO markers.

Theme 2: Qualitative GMO chips

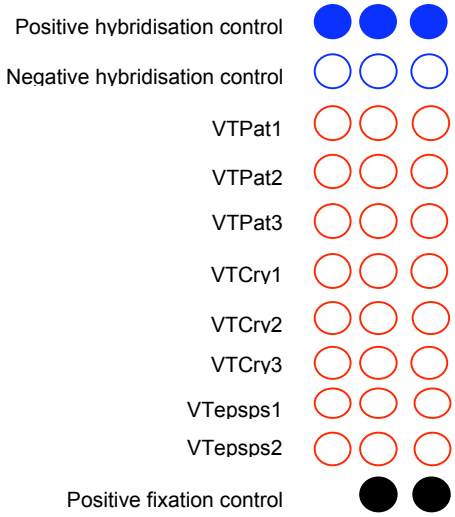
All results concerning this task are in the **annex II under 6.2**.
The consensus primers were tested on genomic DNA of the Bt11, Bt176, GA21 and RRS GMO. The figure 2 in annex II under 6.2 shows that the amplification of Bt11 and Bt176 with the consensus primer of CryAb gene is not specific. The expected fragment size is around 200 bp. This fragment is not predominant for Bt11. The amplification of Mon810 with these primers has not yet been tested on genomic DNA but the PCR on plasmid gives a unique band at 200 bp (figure 3).

RRS and GA21 were amplified with the EPSPS consensus primers. As shown in figure 4 in annex II under 6.2, a unique band at the expected size (180 bp) is amplified for GA21. For RRS, the amplification is not as specific. Indeed, an unspecific amplicon more than 1Kb in size is amplified. Further, the expected 180 bp amplicon migrates approximately as a 210bp amplicon. At the moment, we are cloning and sequencing this PCR fragment to make sure it could be the good target.

The consensus primers of the PAT gene were designed and tested on genomic DNA of Bt11 and a plasmid of T25. The Bt11 amplification of the PAT gene from genomic DNA is very weak (data not shown). The amplification of the T25 plasmid with these primers gives a unique band at the expected size of 160 bp (figure 5).

As we see, all the consensus PCR are not specific enough. There is still a lot of work to be done in a attempt to improve the consensus PCR on genomic DNA. However, at the moment, biotinylated PCR on plasmids can be done to produce the amplicons needed to start the hybridisation tests on the biochips.

For each of the 6 GMO available, capture probes have been designed. We have also chosen capture probes for not yet available GMO like Topas19/2, RF3 and Falcon GS to check the cross reactivity. The names of the capture probes as well as their theoretical specificity are shown in table 2. After the selection of the capture probes, they were spotted on glass slides. The design of the first biochips is represented here after.

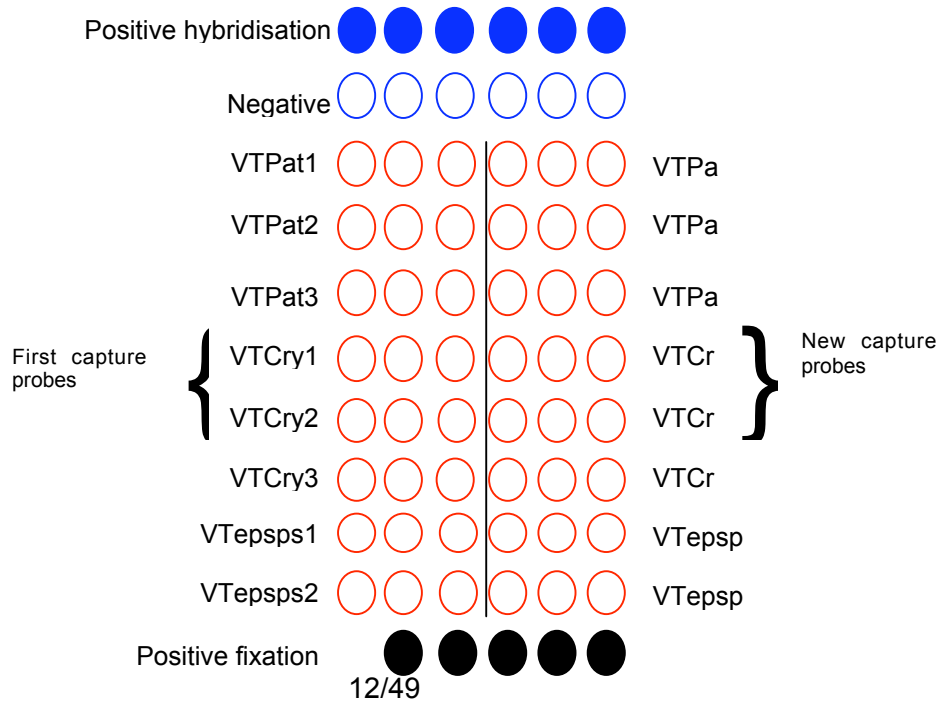


On the design, we can see several controls: a fixation control to check for spotting efficiency, a positive hybridisation and a negative hybridisation control to check for contamination.
After running the PCR with the biotinylated dUTP, most frequently on plasmids, the amplicons were hybridised on the biochips. As shown in figure 6, the intensity of the different spots is very weak. The weakness of the spots doesn't allow us to check for cross-reactivity.

In order to solve this problem, different parameters were tested: hybridisation temperature, stringency of washings, concentration of the capture probes spotted on the slides and incorporation of biotinylated dCTP in the PCR.

Since these different tests did not allow an increase in the intensity of the spots, new capture probes were designed and ordered for each GMO. Unfortunately, for most of the capture probes, the problem persisted.

Finally, new consensus PCR were carried out by changing the batch of biotinylated dUTP. These amplicons were then hybridised on glass slides and the results were compared with the hybridisation of the previous PCR products. All capture probes were spotted on the glass slides. The design of the biochips is represented in figure here below.



This comparison allowed us to call into question the first biotinylated dUTP batch. The hybridisations were then carried out again with the new PCR products for each GMO. The results of these hybridisations are shown in figure 7. In a general way, we can observe that the signals with the first set of capture probes are stronger.

T25 and Bt11(PAT) detection

The amplified product not only hybridises on the specific capture probes (VTPat1 and VTPat4) but also on capture probes that should be specific to Topas19/2 and Falcon GS. For Bt11, we can also observe a slight hybridisation on VTPat3 capture probe.

Bt176, Mon810, Bt11 (Cry) detection

These GMO were amplified with the consensus primers of CryAb gene. We can observe a specific detection for each of them.

RRS and GA21

For RRS, there is no specific detection. We can only observe a slight signal on VTepsps1 that should be specific to GA21. For GA21, the amplified product hybridises only on the specific capture probes (VTepsps1 and Vtepsps3).

Theme 3, taskC.2.1 and C.2.2: Influence of DNA purity on real time PCR

All results concerning this task are in the **annex II under 6.2**.

General overview of the real time PCR

Real time PCR offers the possibility to monitor each PCR cycle reaction (principles of the PCR and the real time PCR are described in appendix 1). Monitoring of the whole PCR allows drawing a characteristic curve that could be split in three main phases:

- A baseline where the exponential amplification is not visible
- An exponential amplification phase where the theoretical amplification coefficient is a factor 2, meaning that at each cycle the copy number of an amplicon double.
- A linear and plateau phases.

From thousands runs we do perform for this first year project using two different real time PCR thermocycler (ABI 5700 and ABI 7000), we do observed that the exponential phase could be split in two distinct phases (Figures 3a and 3b). The first one, optional, has erratic features (for example an amplification coefficient more than 2). The second one is the real exponential phase. We do thus split the curves of each experiment in 4 phases:

Phase1 (□1): Baseline (no visible exponential amplification)

Phase2 (□2): optional phase (visible or not) with erratic features for the exponential amplification

Phase3 (□3): real exponential phase

Phase4 (□4): Linear and plateau phases.

Comparison of different amplifications should be performed during the phase 3. However, one should care on the fact that, as comparing numerous curves in phase 3, a common threshold should be set for all curves. We do thus introduce the concept of a "consensus interval" that define the range of fluorescence values within we can really compared the Ct for each curve (Figure 4).

Influence of DNA extraction methods on real time PCR

The efficiency of the different DNA extraction methods tested so far are summarised in table 1 and 2. Only the phenol/chloroform method allows getting enough DNA for reliable OD measurements. All extracted were controlled on agarose gel for their integrity (figure 2). We wondered to explore and compare the amplificability of these different DNA by mean of real time PCR.

Preliminary experiments allow to optimise the real time PCR conditions, to choose the linearized pLec2 plasmid purified on cesium chloride as reference for the amplificability and at least to choose the ABI7000 thermocycler (appendix 2).

The linearized pLec2 plasmid was also used as reference for calibration. From this, it has been determined that the initial concentration set for each DNA engaged in the PCR should be equivalent to 100.000 copies of the linearized pLec2 plasmid. The two parameters that was monitored was the amplification efficiency and the variability of the measure for a same extract.

The amplification efficiency is expressed as an coefficient of amplification (CA) that is the time required to double the fluorescence for each PCR cycle (theoretical CA value is 2). The variability is expressed as a coefficient of variation (CV) based on the number of cycles required to reach the threshold value.

Results in Table 4 demonstrate that outside the "consensus interval", the phenol/chloroform DNA extract treated with RNase A has the higher CA value even when compared to the linearized pLec2 plasmid. When suppressing the RNase digestion, the same extract has the lowest CA value. In the "consensus interval", the HP extract has the higher CA value (close to the one gain outside the "consensus interval", indicating the robustness in the exponential phase for the HP extracts). However, CA values look homogenous for all extracts.
The CV values indicate that differences variability is less relevant than CA for each extract.

Altogether, the results obtained so far demonstrate the importance to work within a particular phase of the exponential amplification in real time PCR as comparing different samples (Phase 3 in figure 3a and 3b in annex III).
We also do demonstrate that, for a define concentration of the soybean DNA (100.000 copies of the soybean haploid genome), it is possible to have a similar efficiency of amplification between genomic DNA and linearized plasmid DNA purified on cesium chloride.
We could observe a positive effect of RNase digestion for the PC method. However, this effect is not correlated for the others methods (reverse effect on HP method).
It has also been possible to demonstrate the ability to amplify by mean of real time PCR DNA collected after HPLC purification.

Theme 3, taskC.2.3: Analytical description of DNA quality

Promoter # 5 carried out all soybean and plasmid extractions. All figures of this task are assembled in **annex IV under 6.2**.
Figure 1 shows an image of all genomic DNA extracts that were tested. Promoter #5 carried out DNA quantification using spectrophotometry.

Separations on a WatersPak DEAE 15 HR column

Three genomic DNA extracts as well as the salmon sperm DNA and the λHindIII digest were loaded on a DEAE 15 HR column. Flow rate was set at 0.5 ml.min^{-1}. Two main observations can be made.
First, extracts loaded on the 15 HR column elute as very broad peaks indicating resolution is rather poor. As an example, we show the elution of the λDNA-Hind III digest (Fig. 2) that is characterised by a mixture of 7 different fragments varying in size from 23 kb down to 525 bp (Fig. 1). None of the fragments could be separated from the others using this type of column.
Second, all three extracts tested (FC-, HP- and KF-extracts) revealed that besides DNA, a lot of PCR-negative molecules eluted from the column. As an example, the elution of 2.2 µg HP-extract with corresponding UV-spectra and PCR assay is shown (Fig 3). The UV-spectra of the peaks at Rt 60.1 min and Rt 71.5 min are poor spectra compared to those of fig 2b.

Separations on a WatersPak DEAE 8 HR column

In an attempt to increase separation of DNA molecules from what we believe to be essentially RNA molecules, a second column (PAK DEAE 8 HR) was set up. Both linear (gradient 8, appendix 1) and step gradients (gradient 11, appendix1) were tried out. In addition soymeal DNA extracts were incubated in the presence of RNase A (Invitrogen) prior to elution.
First, elution of the CsCl-purified pLec plasmid with gradient 8 is shown (Fig 4). Besides the injection peak, a rather sharp peak was observed at Rt 53.9 min. The corresponding UV-spectrum was that of a DNA molecule with a 260/280 ratio of 1.99. It should be mentioned that, despite the CsCl purification, significant amounts of RNA were still present in the extract prior to elution as could be confirmed by RNase and DNase digestion (not shown).

Despite the higher resolution of the column, the PC-extract still eluted as one major peak. We therefore decided to elute soymeal DNA extracts with a step gradient.
Gradient optimization was performed with a PC-extract. The final gradient (gradient 11) was tried on the circular pLec plasmid. In the corresponding chromatogram, three peaks can be distinguished. Initially, these were thought to correspond to different plasmid conformations such as the relaxed and supercoiled one (Fig 5). Further analyses, however, showed that the peak at R_t 31 is mainly composed of RNA, whereas the last peak contains the plasmid.

Application of gradient 11 on 6.4 µg of a PC-extract is shown in figure 6. Four main peaks are visible. DNA was believed to accumulate in peak with Rt 50.8 as PCR analysis revealed positive fractions between Rt 48 and Rt 56. However, no homogenous DNA fraction was obtained, as a large part of peak 50.8 contained RNA. This was clearly shown when RNA digestion was carried out prior to elution

(Fig 7). Only traces of the peaks with Rt42 and Rt 50.8 remain visible, whereas a lot of degraded RNA molecules eluted early between Rt 15 and Rt 35 min.
PCR positive fractions were still observed between Rt 44 and Rt 55 min. When a DNase digestion was carried out on 6.4 µg PC-extract, all peaks remained intact except for peak Rt 51 (not shown). The latter was, thus, degraded both by RNase and DNase digestion.

In general, we can conclude that for all three extracts a significant amount of RNA was still present in the extract and this was especially true for the PC-extract. This was already suggested by the agarose gel analysis (Fig 1) and could be confirmed by AEC. This means that DNA quantification by using a spectrophotometer is rather unreliable. Actually, we estimated that the amount of DNA present in the PC-extract lies beyond 10% of the spectrophotometrically measured value. This would mean that only 0.64 µg of the total PC-extract loaded onto the column (6.4 µg) could be attributed to DNA molecules.
A second conclusion is that elution profiles were very different from one extract to the other and that genomic DNA, as judged by positive PCR signals, eluted in one, two or even three different peaks (not shown). This is in contrast to the elution profiles of short-chained DNA such as salmon sperm DNA or pLec plasmid that eluted in sharp, symmetrical peaks.
Finally, it seems worthwhile mentioning that an unknown amount of nucleic acid material remained in the HPLC chain after the injection and that it gradually detached from the column during subsequent blank elutions.
PCR analysis of fractions collected from 5 subsequent blanks revealed that positive signals were still detected in fractions of the fifth blank, when the latter were concentrated and desalted prior to PCR analysis. More attention will be drawn to this problem in future experiments.

Separations on the Waters Gen Pak Fax column

As mentioned earlier, the Gen Pak Fax column is composed of small non-porous particles that allow for increasing resolution as compared to previous columns. This was tested on the l HindIII digest using step gradient 14 (appendix 1). As shown in figure 8, the extract eluted in 6 different peaks. The nature of these peaks was determined by concentrating corresponding fractions on centricon 30 filters (Millipore) and analysing them by gel electrophoresis. Peak 1 was not collected but was supposed to contain the 525-bp fragment; peak 2 and 3 (collected in the same fraction) were composed of fragments 2027 + 2322 and 4361, respectively; peak 4 of the 6557-bp fragment, peak 5 of fragment 9416 and peak 6 of the longest 23120 bp-fragment.

The most promising separation of the PC-extract was achieved with step gradient 17 but DNA still-co-eluted with RNA in the last peak with Rt = 36.0 min (not shown). No other genomic DNA extracts were separated on this column.

Finally, we also tried to quantify the amount of DNA that remained in the column after elution of the PC-extract. By integrating the peak surfaces (UV signal), we could determine that a PCR-positive peak was present in the subsequent blank injection, which equalled 15% of the DNA peak area of the PC-extract.
In the following experiments we decided to introduce a triple washing step consisting of three passages from low to high salt followed by one or two blanks between every

DNA extract chromatography. By applying this protocol, blanks following the washing step were reduced to a flat line except for the injection peak (not shown).

Separations on the Dionex DNA Pac PA-100 column

The Dionex DNA Pac PA100 column was selected for its excellent resolution and for its functional separation in a broad pH range.
The chromatogram in figure 9A represents the separation of 6.4 µg of the PC-extract with gradient 17. Again, several peaks were observed that clearly corresponded to the different salt steps induced in the gradient. The last peak (Rt 35), however, eluted as a double peak with positive PCR fractions eluting in the second half. The doubling of the peak was also shown for the KF-fraction (Fig 9B) and the HP-fraction (Fig 9C). When a DNAse-digest was carried out on each of these extracts prior to elution (Fig 10), the second half of the double peak -indicated by black arrows in figures 9A to 9C for PC-, KF- and HP-extracts respectively- disappeared.
When RNase digestions were carried out, the double peak (Rt = 35 and 36,5 min) was reduced by 90 to 95% for all extracts except for the HP-extract. The latter preserved almost the totality of the peak at Rt = 36.5 min (Fig. 11).

By replacing KCl with $NaClO_4$, and maintaining a step gradient, we were able to slightly increase the resolution of the double peak for the PC-extract (Fig.12).

At this stage, a fourth DNA extract was analysed, namely the CTAB extract. In contrast to previous extracts, chromatography of this extract resulted in only two small peaks with Rt = 27 and Rt = 31,2 min (Fig 13). Surprisingly, PCR positive fractions were already present from Rt = 27 min whereas positive PCR fractions for all other extracts started at Rt = 31 min. This might be due to the average size of DNA fragments. Actually, when sonicated PC-extracts are loaded on a similar column, PCR positive signals started at Rt = 27 min.
When DNase digestion was carried out on the CTAB-extract prior to injection the peak at Rt = 31.2 min almost completely disappeared suggesting it is mainly composed of DNA.

Influence of DNA quality on real time PCR (promoter #5)

The purpose is to distinguish among different impurities that can affect the real time PCR. Before answering this purpose, it was first obvious to see the amplificability of a DNA template after its purification on HPLC column. The experiment was performed with the pLec2 DNA. The results showed in figure 14 demonstrate that fractions containing that DNA gave a specific amplicon. Fractions without DNA collected before or after the DNA peak failed to be amplified. Interestingly, fractions preceding or following the DNA peak (with potential trace amount of DNA) were also amplifiable but the signal obtained was lower. There was a correlation between the retention time on the column and the efficiency of the amplification. The less is the plasmid copy number, the more the amplification is delayed (“dilution effect”).

5.Future prospects and future planning

Theme 1: Production and deposit of plasmid reference material.

There is a huge interest all over control laboratories to get reliable reference material for GMO analyses. We thus plan in the coming year to further finalise the deposit of existing GMO plasmid to BCCM. For this purpose, it has still to make all GMO constructs clones available in pUC18 to fulfil the requirement of the scientific dossier.

The GMO market is going to increase qualitatively but also quantitatively. It is of importance to anticipate coming rules regarding new GMOs. We thus plan to develop new screening markers to be able to trace non authorised GMO in EU.

Plasmid reference material has to be proved equivalent for quantitative analyses compared to the genomic DNA extracted from raw seeds reference material. We should then perform statistical validation of correspondence between IRMM standards (genomic DNA) and copies number (using plasmids) (collaboration between promoters #1, #2 and #5).

Theme 2: Qualitative GMO chips

The qualitative biochip has to be upgraded for the species-specific detection and also for the detection of the RRS event. Once ready these biochips should be tested in collaboration between the ISP (promoter #1) and the FUNDP (promoter #3) for different matrices.

Theme 3, taskC.2.1 and C.2.2: Influence of DNA purity on real time PCR

In a first step, we will perform systematic comparison between different fractions of each kind of extract after HPLC processing (control salt concentration because it may different for each fraction) in other to check influence on the efficiency on PCR.

The influence of DNA purity was tested here for soybean flour. The same tests should be performed on other matrices (maize or some other more complex matrices)

Addition of pure components in reference DNA has also to be assessed for its effect on real time PCR (plasmid and/ or genomic DNA)

Theme 3, taskC.2.3: Analytical description of DNA quality

The chromatogram of the DNA separation is strongly dependent on the type of extract that was used. This is not surprising considering that these extracts are “contaminated” by varying amounts of RNA. In this context it seems worthwhile mentioning that despite the RNase treatment of some extracts (KF and CTAB) during the extraction protocol, significant quantities of RNA were still present in the extract.

In the near future, all DNA-extracts will be systematically treated with RNase during extraction. This should strongly improve DNA purification.

Secondly, analysis of all DNA extracts (before and after chromatography) will be carried out by pulsed field gel electrophoresis (CHEF, Bio-Rad) so that the exact size of all DNA fragments will be determined more precisely.

Third, recovery experiments will be carried out to determine the amount of DNA that remains in the HPLC system upon chromatography. Preliminary experiments indicated that 60 to 70 % of DNA was eluted during the first run and another 10% during the subsequent blank run. More precise determination of recovery of DNA could be performed using a DNA-specific dye such as PicoGreen (Molecular Probes).

The amounts of DNA should be measured more accurately to allow reliable comparison of the raw and the HPLC purified DNA in real time PCR experiments (promoter #5) and to enable the identification of chemical descriptors.

Finally, if components of the early eluting peak should interfere with real time PCR measurements, its composition will be analysed more carefully. Preliminary reversed phase chromatography experiments to separate the different components have already been carried out.

6. Annexes

6.1 References

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6.2 Detailed results

ANNEX I: Theme 1: Production and deposit of plasmid reference material

Table1: overview of GMO construct-specific and species-specific plasmids (promoter #1)

		Gene Trait sequence	Characteristic of Gene Trait	Bank Access Number	Seq. Positions	Insert size	Cloning vector	Available in pUC 18
1	Bar	bar present in Bt11 en T25	phosphinothricin tolerance	Home	1-550	550	Topo-TA	No
2	Mais	zein	Zea mays 10kDa Zein gene	M23537.1	1194-1470	277	Topo-TA	No
3		zein	Zea mays 10kDa Zein gene	U25674	1-531	531	Topo-TA	No
4		invertase	invertase gene in Zea Mays	U16123	507-1317	810	/	No
5	lectine	lectine	soya lectin Le1(*glycin. Max.)*	K00821	934-1881	948	Topo-TA	Yes:pENGL-02-001
6		lectine	soya lectin Le1(*glycin. Max.)*	K00821	1114-1736	622	Topo-TA	No
7		lectine	soya lectin Le1(*glycin. Max.)*	K00821	1559-1736	178	Topo-TA	No
9	cry 9C	cry9C	Starlink cry9cgene sequence	Home	18-941	924	Topo-TA	No
10		cry9C	Starlink cry9cgene sequence	Home	511-1530	1019	Topo-TA	No
11		cry9C	Starlink cry9cgene sequence	Home	960-1878	918	Topo-TA	No
12		cry9C	Starlink cry9cgene sequence	Home	1063-1243	180	pGEMTeasy	No
13		border sequence	Starlink cry9cgene sequence	Home	confidential	178	pGEMTeasy	No
14	Oilseed rape	PEP3-PECase	gene for phosphoenolpyruvate carboxylase from Brassica napus	D13987	2601-3889	1288	/	
15		PEP3-PECase	gene for phosphoenolpyruvate carboxylase from Brassica napus	D13987	2652-3637	1005	/	
16		border sequence	MS8 border sequence	Home	confidential	280	pGEMTeasy	No
17		border sequence	RF3 border sequence	Home	confidential	215	pGEMTeasy	No
18		cruciférine	cruciférine cruA for Colza	X14555	791-1183	394	pGEMTeasy	No
19	Virus	CRT	reverse transcriptase gene (ORFV) from Cauliflower mosaic virus	Home	Publication	191	Topo-TA	Yes:pENGL-02-002
20		CAMV	inclusion body matrix protein of CAMV	Home	Publication	383	Topo-TA	Yes:pENGL-02-003
22	promotor	promotor CamV 35S	35S-promotor of cauliflower mosaic virus (CaMV)	V00141.1	7249-7395	147	Topo-TA	No
23	terminator	terminator nos	Agrobacterium tumefaciens gene encoding nopaline synthetase	V00087.1	1885-2002	118	Topo-TA	No

table 2: Overview of GMO event-specific plasmids (promoter #2)

	FIRST CLONE				pUC18 CLONE	DEPOSITED PLASMID LMBP-BCCM
	Species/GMO event	pDNA	Inserted sequence	Insert length (bp)	NAME *italic=planned*	
JUNCTIONS READY	RR soybean	pAS104	p-35S border	359	pENGL-01-001	LMBP4356 (pAS104)
		pCM7	p-35S border	422	*pENGL-01-012*	
		pAM1	t-NOS border	928	pENGL-01-003	
	Ms8 rapeseed	pCM1	left border	792	*pENGL-01-006*	
		pCM5	right border	629	*pENGL-01-010*	
	Rf3 rapeseed	pCM6	right border	513	*pENGL-01-011*	
	GT73 rapeseed	pCM8	left border	512	*pENGL-01-013*	
	CBH351 (StarLink) maize	pCM2	t-NOS border A	549	*pENGL-01-007*	
		pCM3	t-NOS border B	887	*pENGL-01-008*	
	Mon810 maize	pCM4	p-35S border	901	*pENGL-01-009*	

Table 3: Overview of species-specific plasmids (promoter #2)

	FIRST CLONE				pUC18 CLONE	DEPOSITED PLASMID LMBP-BCCM
	Species	pDNA	Inserted sequence	Insert length (bp)	NAME *italic=planned*	
ENDOGENOUS GENES	WT soybean	pAS106	endogenous *lectin* gene fragment	118	pENGL-01-002	LMBP4357 (pAS106)
		pCM9	endogenous lectin gene fragment	795	*pENGL-01-014*	
	WT maize	pCM10	endogenous zein gene fragment	655	*pENGL-01-015*	
FILL-IN READY	WT soybean	pAM2	wild type - preinsertion locus (p35S site)	470	pENGL-01-004	
		pAM3	wild type - preinsertion locus (tNOS site)	773	pENGL-01-005	

Scientific report

1. General information
- Name of plasmid: **pENGL-nn-xxx**
- Constructor name
- Constructor date
- Literature reference(s) of the pENGL-plasmid
- Deposit date
- BCCM-LMBP Accession number

2. Host properties
- Scientific name of host organism
- Genetic description of host
- Literature reference(s) of host
- Culture conditions (medium, growth T, resistance markers added)

3. Cloning vector features

Will be pUC18, described fully in BCCM; so refer to BCCM or refer to the JRC-IRMM if this lab has delivered the plasmid.

4. Cloned DNA fragment(s)
- Gene trait sequence(s)
- Characteristic of gene trait(s)
- Accession number(s) in databases (EMBL/GENBANK)
- Literature reference(s) of cloned DNA fragment(s)
- Length of cloned DNA fragment(s)

5. Resulting plasmid

5.1. Quality/authenticity testing
- Clarify choice of sequence and size of DNA fragment
- Origin of template DNA
- DNA extraction method
- PCR specifications: Taq DNA polymerase, reaction mixture, conditions
- TA-cloning:
  - ➢ Ligation and transformation: kit, reaction mixture, conditions
  - ➢ Screening/selection criteria of clones:
    (1) PCR
    (2) restriction analysis (enzymes, reagents, conditions)
- pUC18 cloning:
  - ➢ Digestion of first plasmid: enzyme, reaction mixture, conditions
  - ➢ Ligation and transformation: kit, reaction mixture, conditions
  - ➢ Screening/selection criteria of clones:
    (1) PCR
    (2) restriction analysis (enzymes, reagents, conditions)
    (3) sequencing

5.2. Applications
- Qualitative PCR assay: primers, length of amplicon, PCR reaction mixture, conditions
- Quantitative PCR assay: primers, TaqMan probe, length of amplicon, PCR reaction mixture, conditions

ANNEX II: Theme 2: Qualitative GMO chips

Figure 1: Overview of the biochips technique

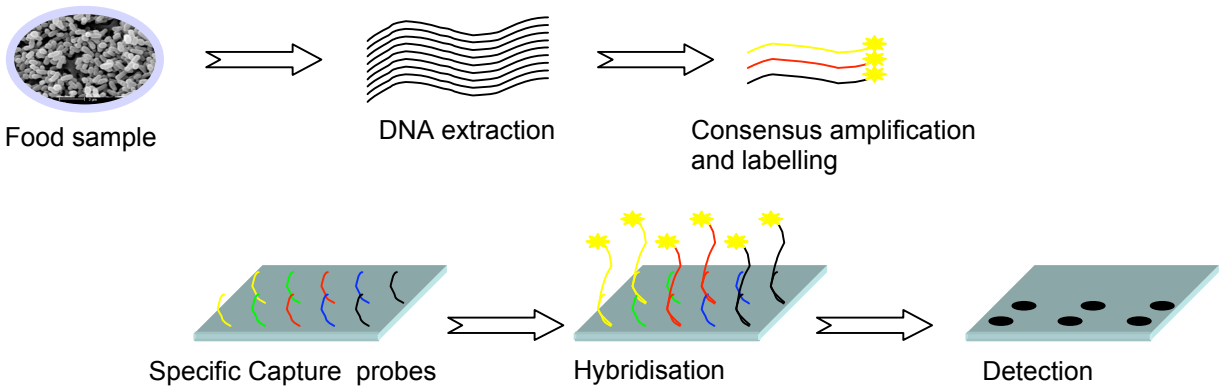


Table 1: description of primers set, gene detected and events for biochips screening

Primers set	Genes	Event
VPpat1-VPpat2	PAT	T25, Bt11
VPepsps1-VPepsps2	EPSPS	GA21, RRS
VPcry1-VPcry2	CryAb	Bt11, Bt176, Mon810

Table 2: name of the different capture probes and their theoretical specificity.

Event	Capture probes
T25, Bt11	VTPat1
Topas19/2, Falcon GS	VTPat2
RF3	VTPat3
GA21	VTepsps1
RRS	VTepsps2
Bt176	VTCry1
Mon810	VTCry2
Bt11	VTCry3

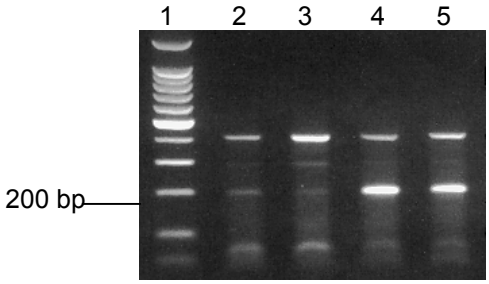


Figure 2: PCR amplification of Bt11(lanes 2 and 3) and Bt176 (lanes 4 and 5) genomic DNA with CryAb consensus primers. Lane 1 stands for the molecular weight marker.

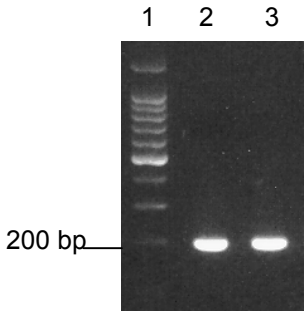


Figure 3: PCR amplification of plasmid Mon810 with CryAb consensus primers (lanes 2 and 3) Lane 1 stands for the molecular weight marker.

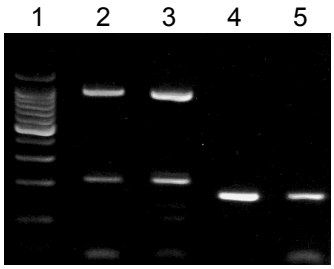


Figure 4: PCR amplification of RRS (lanes 2 and 3) and GA21 (lanes 4 and 5) genomic DNA with EPSPS consensus primers. Lane 1 stands for the molecular weight marker.

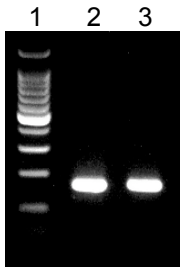


Figure 5 : PCR amplification of plasmid T25 (lanes 2 and 3) with PAT consensus primers. Lane 1 stands for the molecular weight marker

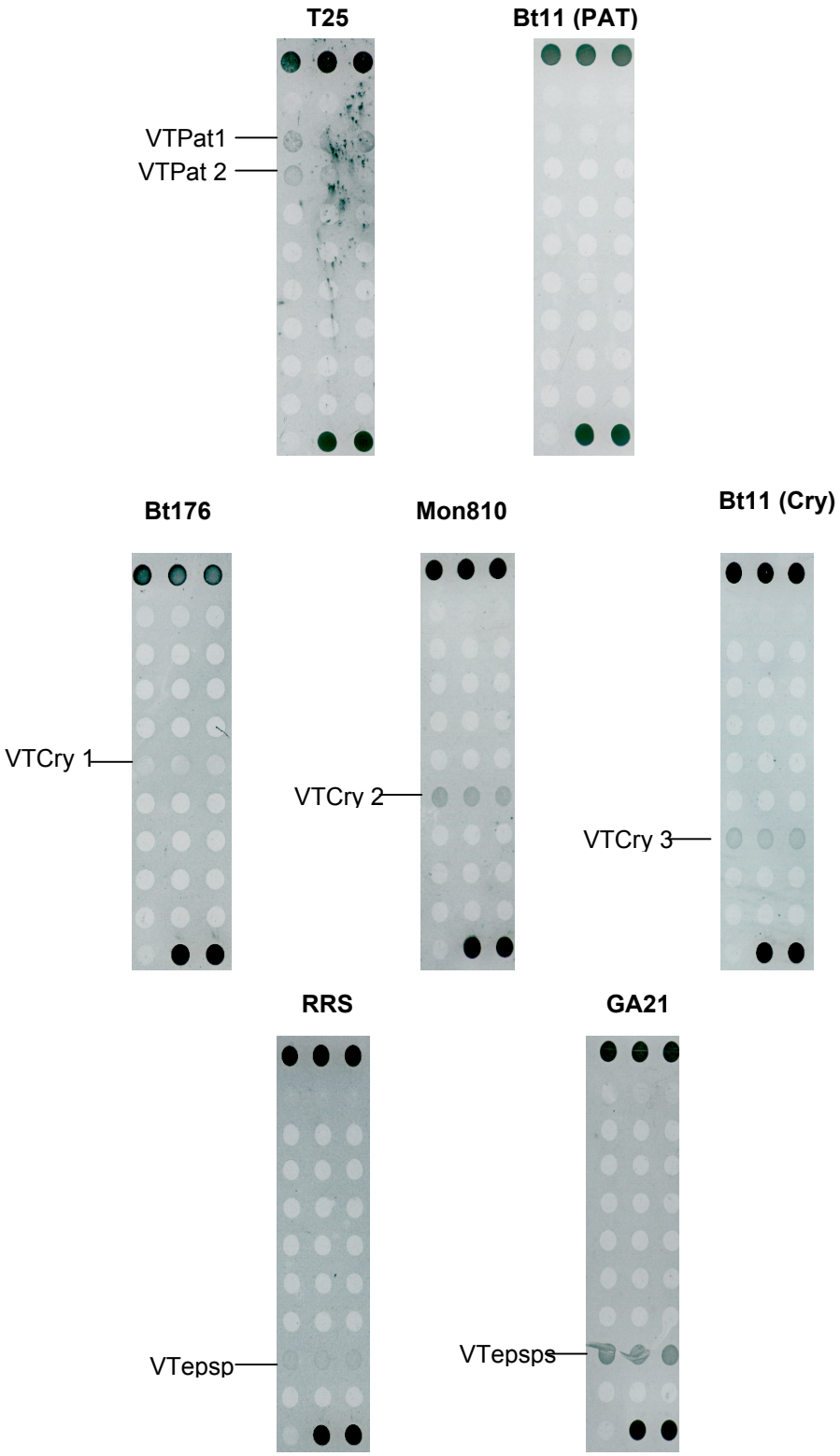


Figure 6 : Hybridisation patterns of T25, Bt11, Bt176, Mon810, RRS and GA21 after PCR amplifications.

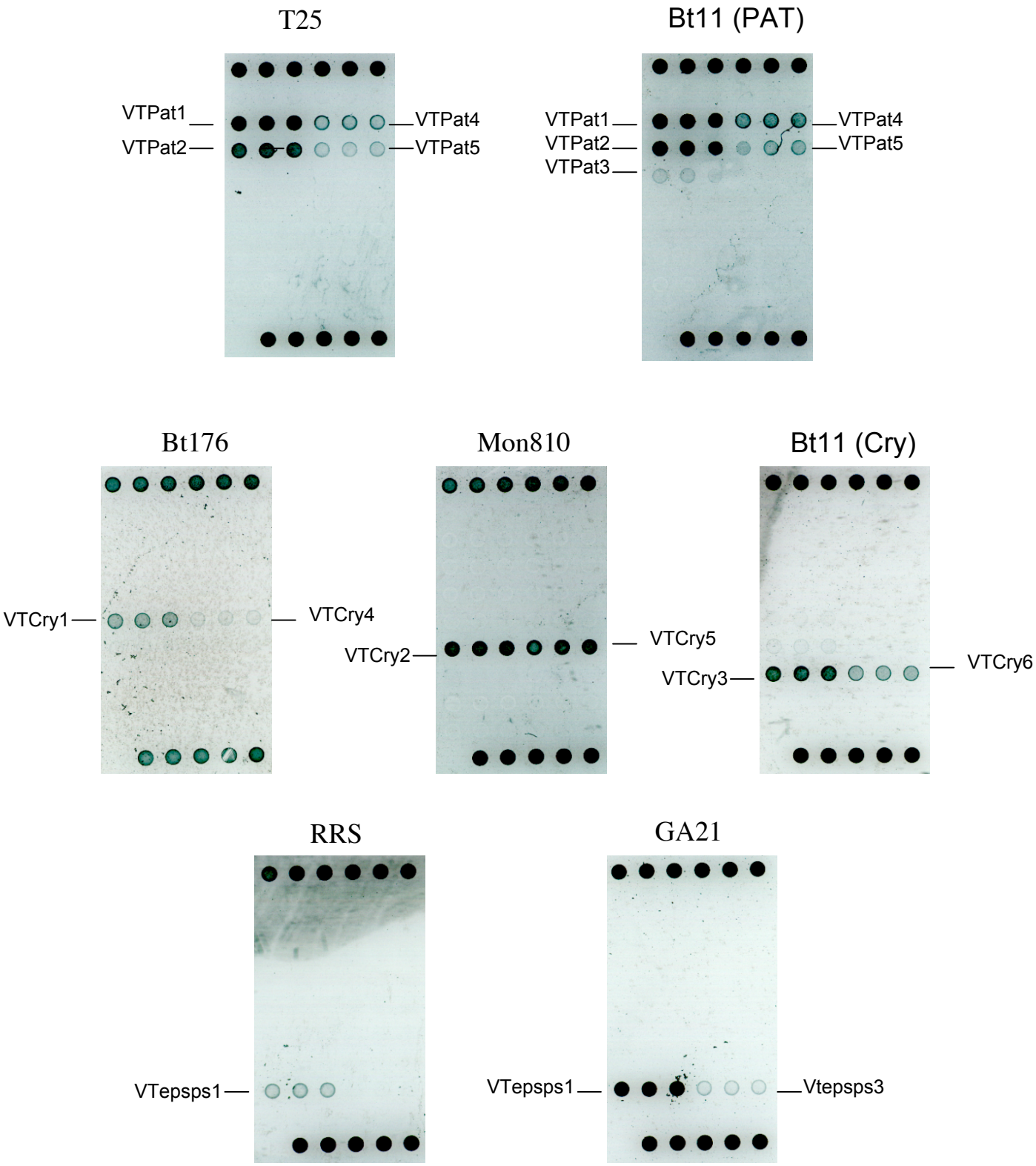


Figure 7: Hybridisation patterns of T25, Bt11, Bt176, Mon810, RRS and GA21 after PCR amplifications with the new batch of biotinylated dUTP.

Annex III: Theme 3, taskC.2.1 and C.2.2: Influence of DNA purity on real time PCR

Table 1: Overview of different DNA extraction methods and their characteristics

Principle	Method	Rnase A treatment	Abreviation	Organic Solvents	Possible automatisation	Time
Sequential precipitation	CTAB	Yes	CTAB	Yes	No	5 to 6h
	Phenol-Chloroform	No	PC	Yes	No	3 to 4h
		Yes*	PC+Rnase			
Affinity resin on minicolumns	High Pure PCR Template Roche	No	HP	No	No	2 to 2h30
		Yes*	HP+Rnase			
Affinity resin on magnetic beads	Promega For Food kit	Yes	PFF	No	Yes	1 to 1h30
	ThermoLifeSciences kit	No	TLS	No	Yes	45' to 1h
* doesn't use RNAse A in basic protocol						

Table 2: assessed yeald of DNA extractions (electrophoresis and OD data)

Method	Yield
PC	High
HP	Mean
CTAB	Low
PFF	Low
TLS	Low

Table 3: OD data about PC and PC+RNase extracts

Extract	OD units/100mg flour	OD 260/ OD 280	µg nucleic acids / 100mg flour (assessment)
PC	4,05	2,20	
PC+Rnase	1,51	1,75	75,55
OD PC/PC+Rnase	0,37		

Calculated fractions of DNA and RNA

	Value	Explanation
µg DNA	75,55	1,51x40 [2]
OD due to dsDNA	37,00%	1,51/4,05
OD due to RNA (DNAss)	63,00%	(100-37)%
µg RNA (and ssDNA)	101,60	(4,05-1,51)x40 [2]
µg nucleic acids	177,15	75,55+101,6
DNA fraction	**43,00%**	(75,55/177,15)x100
RNA (& ssDNA) fraction	**57,00%**	(101,6/177,15)x100

Table 4: Comparison of real time PCR kinetics from different DNA extracts (efficiency and variability analysis)

Relative fluorescence level Range (Rn)	"Nonconsensus interval" Rn : 0,025-->0,1		"Consensus interval" Rn : 0,1-->0,2	
Sample name (4 replicates per sample)	Efficiency CA (cycles)	Variability CV(%)	Efficiency CA (cycles)	Variability CV(%)
pLec 2 plasmid (10+5 cop.)	1,99	2,92	1,86	2,87
Linear pLec2 plasmid (10+5cop.)	1,86	0,53	1,86	0,47
CTAB extract + Rnase (CTAB)	1,70	1,05	1,90	0,66
Phenol-Chloroform extract (PC)	1,74	0,92	1,83	0,78
Phenol-Chloroform + RNase extract (PC+Rnase)*	2,53	0,35	1,91	0,41
High Pure extract (HP)	2,01	0,52	1,98	0,15
High Pure + RNase extract (HP+Rnase)*	1,89	0,97	1,72	0,37
Promega for Food extract + Rnase (PFF)	1,82	1,12	1,88	0,40
ThermoLifeSciences extract (TLS)	1,85	0,71	1,87	0,16

- « **Nonconsensus interval** » means that all curves are in exponential phase but (at least) one of them presents absurd characteristics (for example Coefficient of Amplification >2).
- « **Consensus interval** » means that all curves are in exponential phase without absurd characteristics (Coefficient of Amplification </= 2).
- **CA** = Coefficient of Amplification = required number of cycles for doubling of fluorescence level (with theoretical CA = 2)
- **CV** = Mean Coefficient of Variation calculated (in this case) for 3 fluorescence levels (0.025; 0.05 and 0.1) for « Nonconsensus interval » and for 2 fluorescence levels (0.1 and 0.2) for « Consensus interval ».
- *No RNase treatment in basic protocol.

Figure 1: Schematic vue of pLec2 plasmid (pCR® 2.1 [11] with introduced Lectin gene fragment)

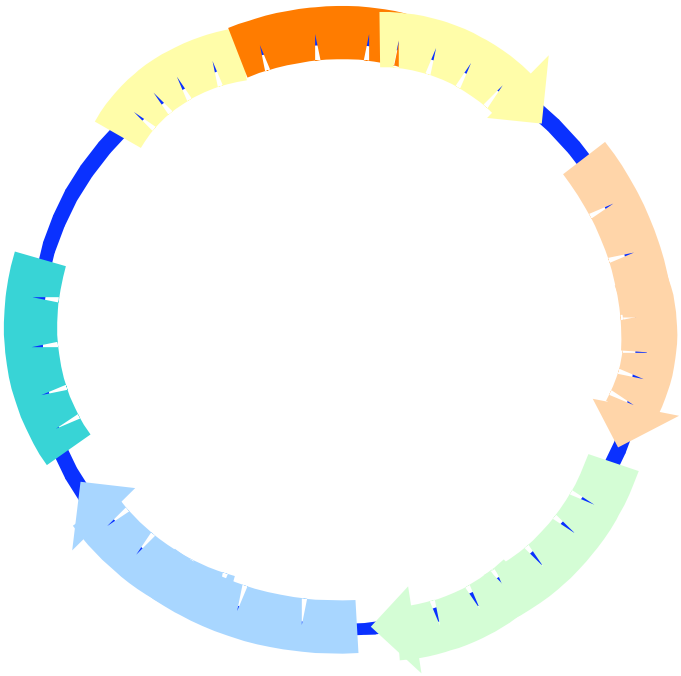


Figure 2: 25 ul of DNA extracted following different methods was loaded on a 0.8% agarose gel. The extraction methods used are PC (lane 2), PC-RNase (lane 3), CTAB-RNase (lane 4), HP (lanes 5 and 7), HP-RNase (lane 6), PFF-RNase (lane 8) and TLS (lane 9). 500 ng Lamda HindIII was loaded on gel as molecular weight markers.

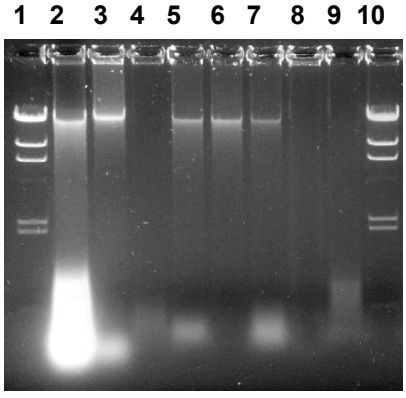
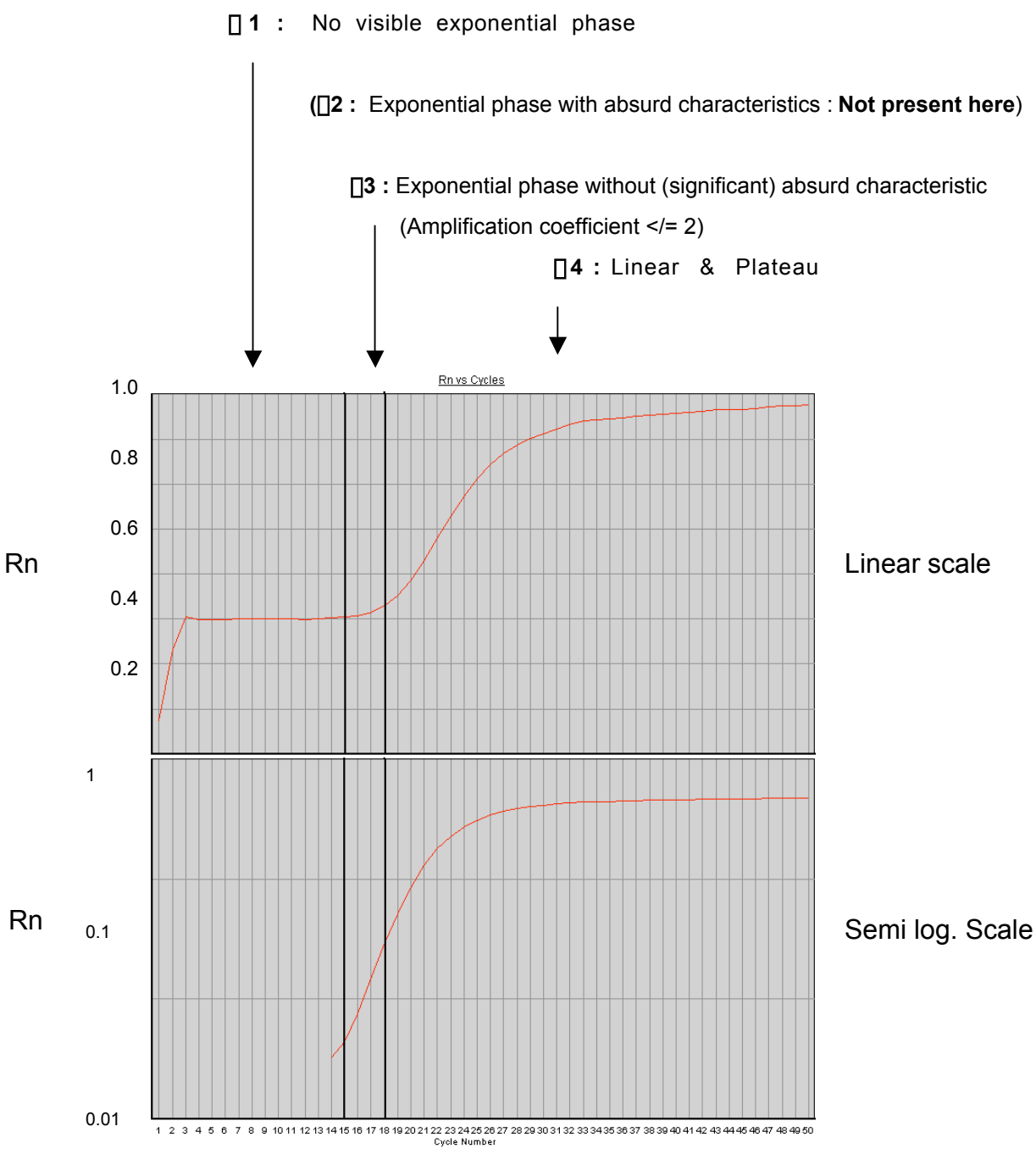
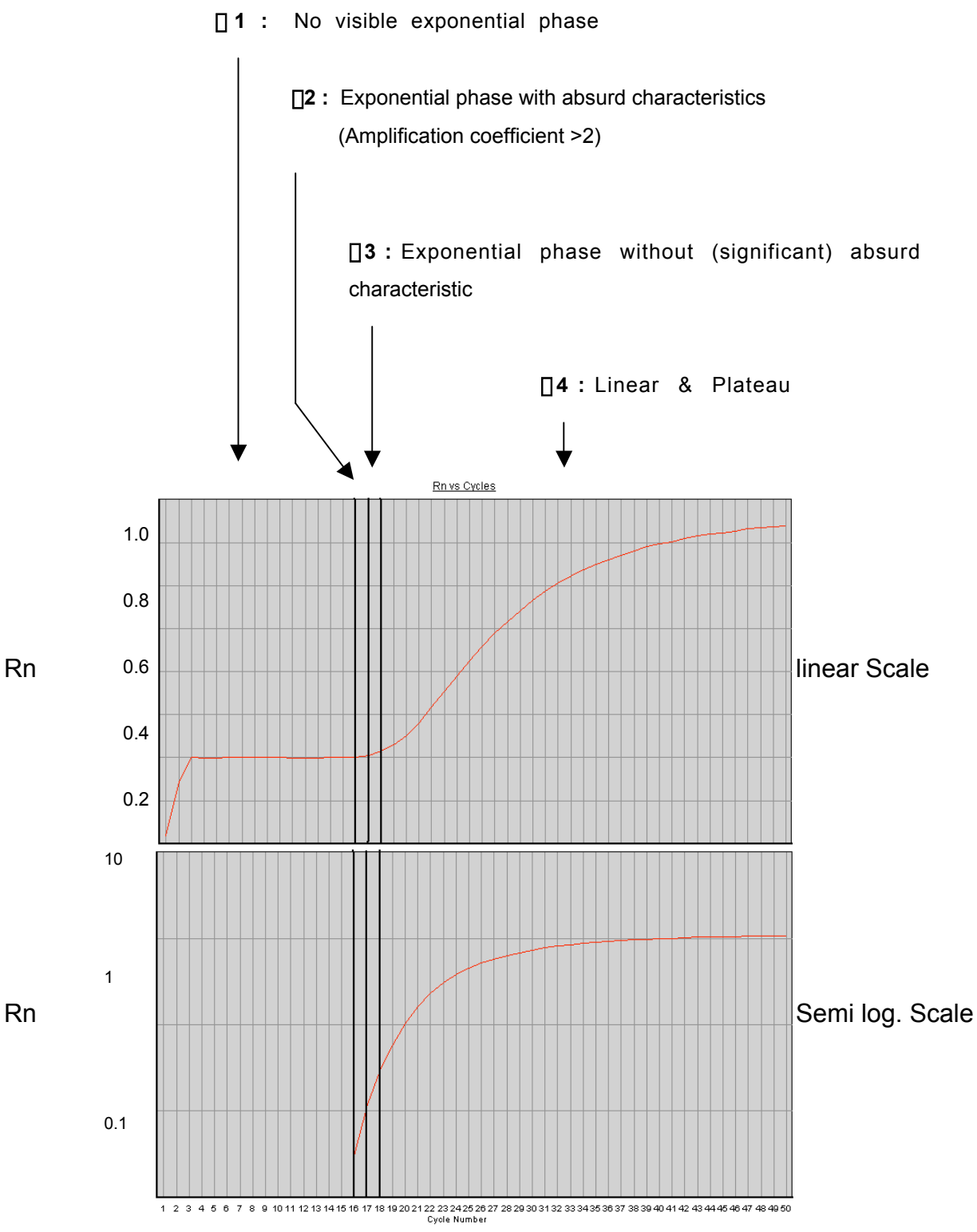


Figure 3a: Illustration of Real-time PCR curve without absurd characteristic



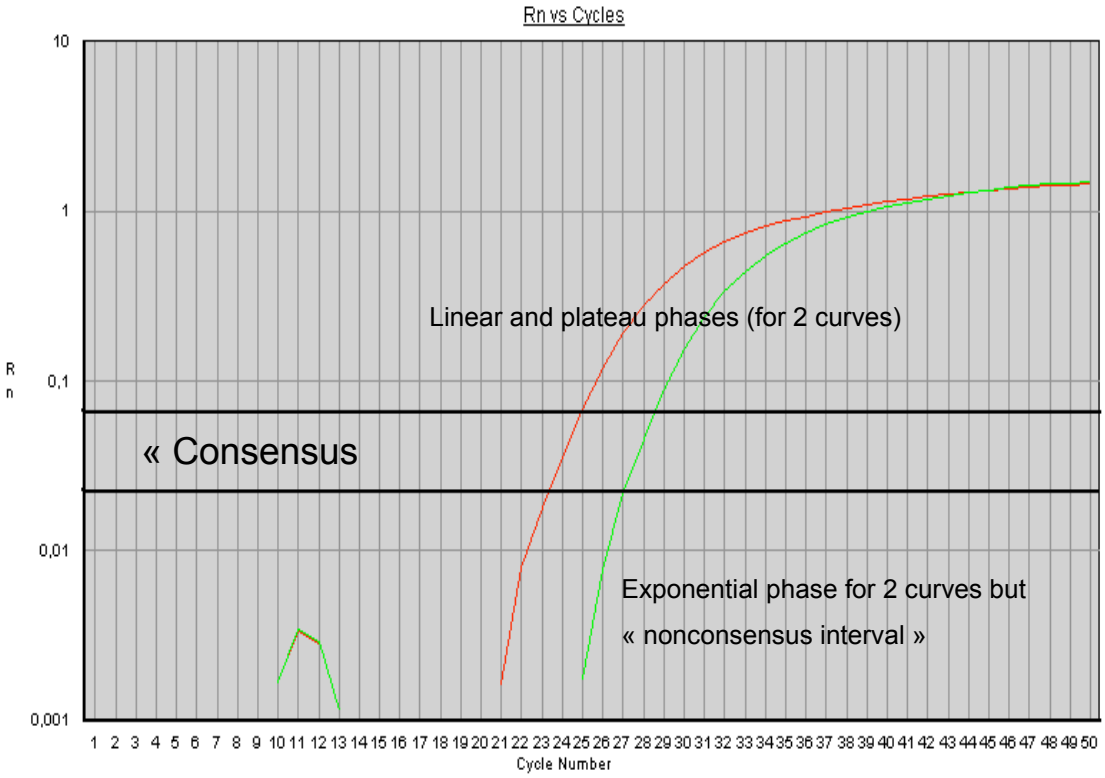
Rn = Relative Fluorescence

Figure 3B: Illustration of Real-time PCR curve withabsurd characteristics



Rn = Relative Fluorescence

Figure 4: Illustration of "Consensus interval"(to compare several Real-time PCR curves) "**Consensus interval**" can be defined as the range of fluorescence levels where all the compared curves are (respectively) in (their) exponential phase but without absurd characteristic. In this figure, the "Consensus interval" is contained between 0.022 and 0.08.



Appendix 1

Appendix 1.A. Principle of the PCR (8)

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing.

1. **The cycling reactions :**

   There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

   1. **Denaturation** at 94°C: During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example: the extension from a previous cycle).

   2. **Annealing** at 54°C: The primers are jiggling around, caused by the Brownian motion. Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the hydrogen bond is so strong between the template and the primer that it does not break anymore.

   3. **Extension** at 72°C: This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, have a stronger attraction to the template, created by hydrogen bonds, than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment.
      The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template)

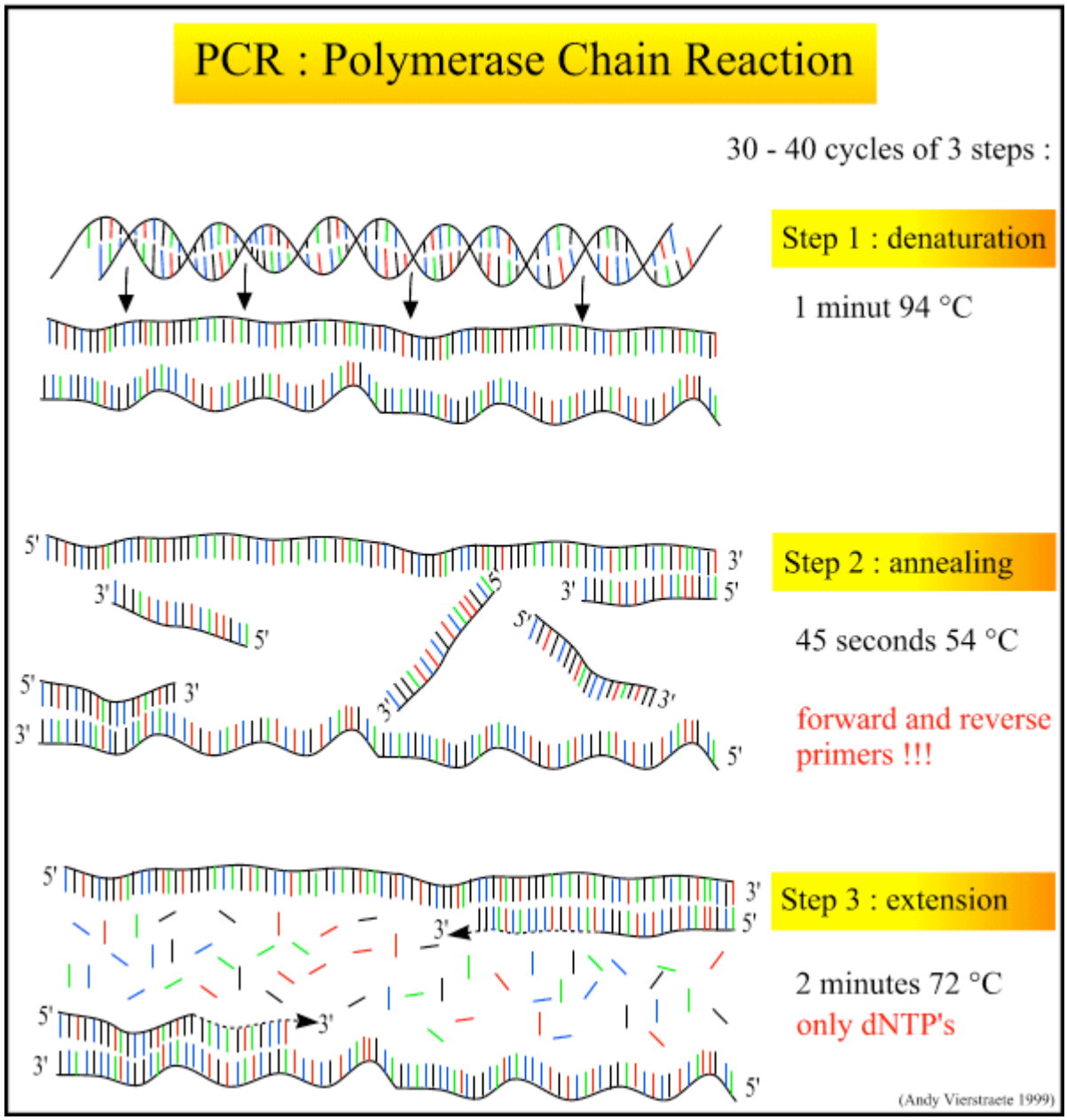


Figure A.3.1: The different steps in PCR.

Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. Suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, three cycles will result in 8 copies and so on.

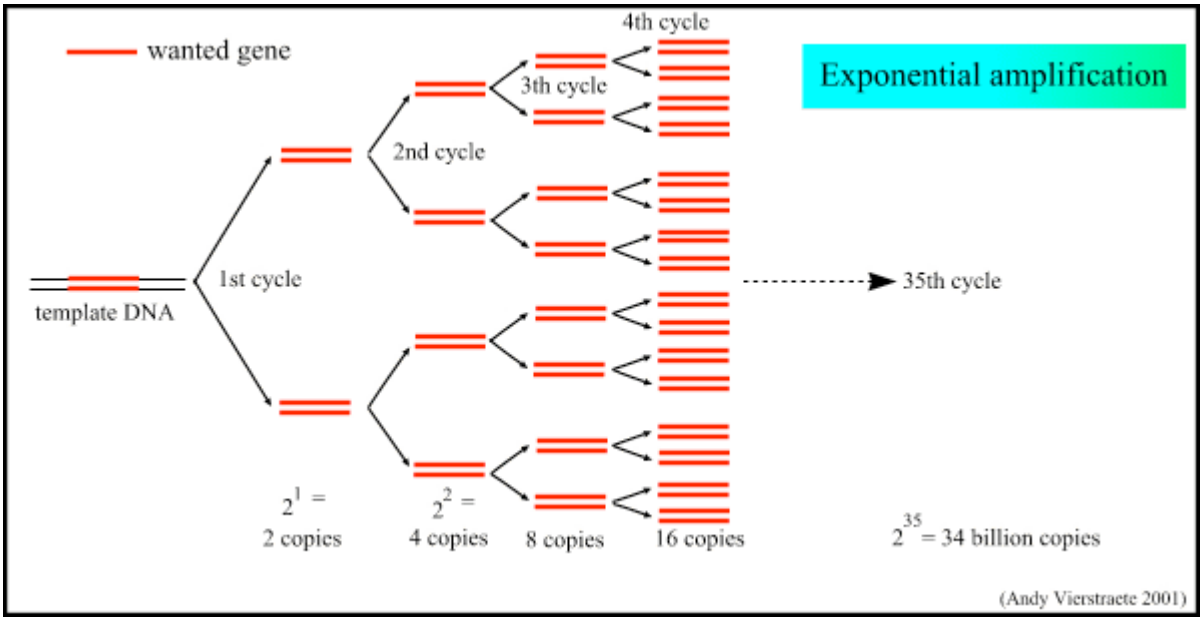


Figure A.3.2: The exponential amplification of the gene in PCR.

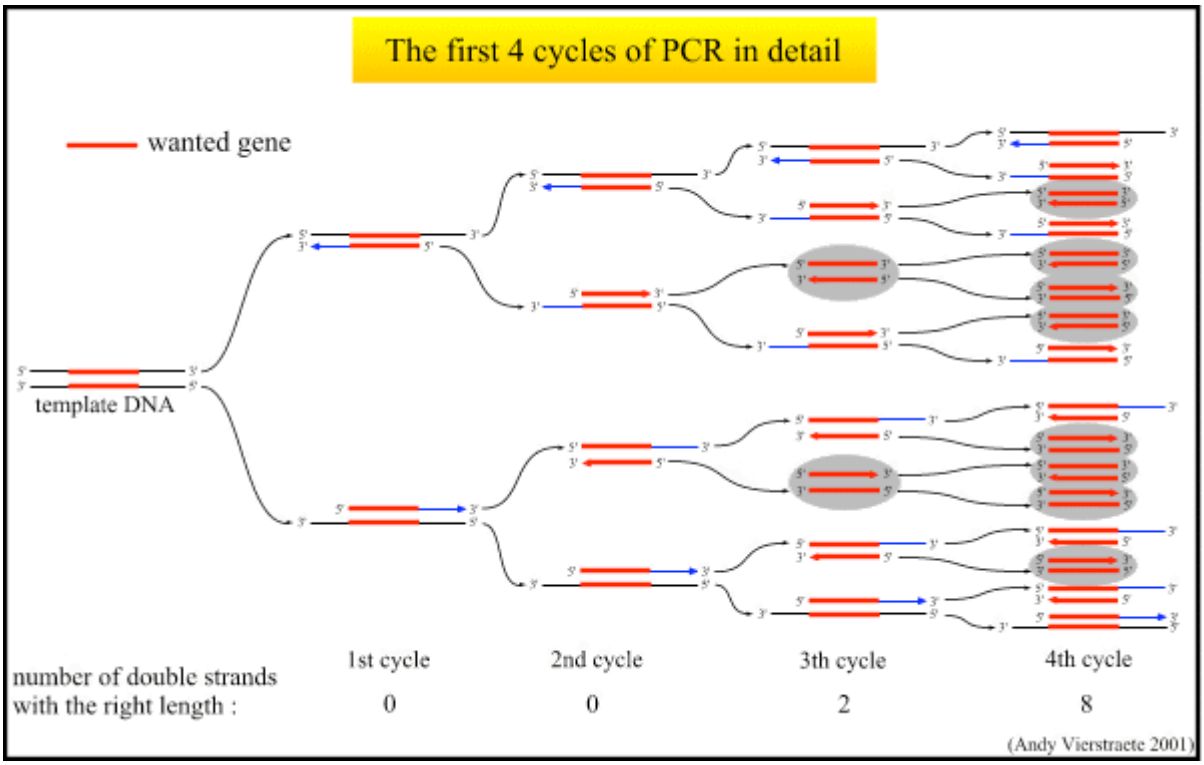


Figure A.3.3: The first 4 cycles of a PCR reaction in detail. In the 3rd cycle, two double strands of the right length are copied (the forward and reverse strand are the same in length). In the 4th cycle, 8 double strands of the right length are copied.

2. **Is there a gene copied during PCR and is it the correct size?**

   Before the PCR product is used in further applications, it has to be checked if:

   1. There is a product formed. Though biochemistry is an exact science, not every PCR is successful. There is for example a possibility that the quality of the DNA is poor, that one of the primers doesn't fit, or that there is too much starting template

   2. The product is of the right size It is possible that there is a product, for example a band of 500 bases, but the expected gene should be 1800 bases long. In that case, one of the primers probably fits on a part of the gene closer to the other primer. It is also possible that both primers fit on a totally different gene.

   3. Only one band is formed. As in the description above, it is possible that the primers fit on the desired locations, and also on other locations. In that case, you can have different bands in one lane on a gel.

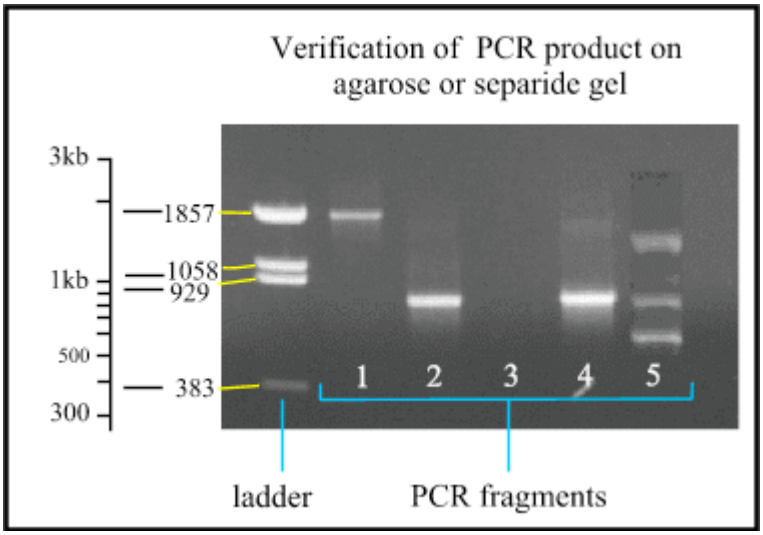


Figure A.3.4: Verification of the PCR product on gel.
The ladder is a mixture of fragments with known size to compare with the PCR fragments. Notice that the distance between the different fragments of the ladder is logarithmic. Lane 1: PCR fragment is approximately 1850 bases in length. Lane 2 and 4: the fragments are approximately 800 bases long. Lane 3 : no product is formed, so the PCR failed. Lane 5: multiple bands are formed because one of the primers fits on different places.

Appendix 1.B. Real-time PCR (9)

1. Real-time PCR chemistry

Real-time systems for PCR were improved by probe-based, rather than intercalator-based, product detection. The principal drawback to intercalator-based detection of PCR product accumulation is that both specific and non-specific products generate signal. An alternative method, the 5' nuclease assay, provides a real-time method for detecting only specific amplification products. During amplification, annealing of the probe to its target sequence generates a substrate that is cleaved by the 5' nuclease activity of Taq DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. This dependence on polymerisation ensures that cleavage of the probe occurs only if the target sequence is being amplified.

The development of fluorogenic probes made it possible to eliminate post-PCR processing for the analysis of probe degradation. The probe is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by Förster resonance energy transfer (FRET) through space. Probe design and synthesis has been simplified by the finding that adequate quenching is observed for probes with the reporter at the 5' end and the quencher at the 3' end.

Figure A.3.5. Diagrams what happens to a fluorogenic probe during the extension phase of PCR. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended. This cleavage of the probe separates the reporter dye from quencher dye, increasing the reporter dye signal. Cleavage removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process. Additional reporter dye molecules are cleaved from their respective probes with each cycle, effecting an increase in fluorescence intensity proportional to the amount of amplicon produced

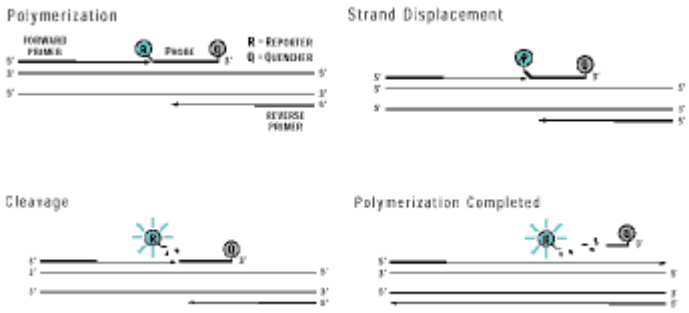


Figure A.3.5. : Principle of TaqMan® probes chemistry (from (10

2. Real-time PCR kinetics

The ability to monitor the real-time progress of the PCR completely revolutionises the way one approaches PCR-based quantification of DNA and RNA. Reactions are characterised by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

Figure A.3.6. shows a representative amplification plot and defines the terms used in the quantification analysis. An amplification plot is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter CT (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. A plot of the log of initial target copy number for a set of standards versus CT is a straight line. Quantification of the amount of target in unknown samples is accomplished by measuring CT and using the standard curve to determine starting copy number.

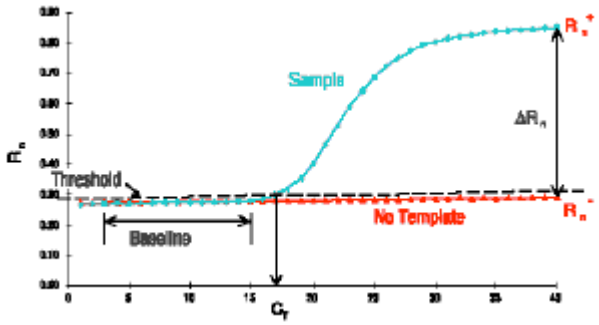


Figure A.3.6.: Example of real-time PCR kinetics (from [10]).

The Polymerase Chain Reaction (PCR) has revolutionised the detection of DNA and RNA. As little as a single copy of a particular sequence can be specifically amplified and detected. Theoretically, there is a quantitative relationship between amount of starting target sequence and amount of PCR product at any given cycle. In practice, though, it is a common experience for replicate reactions to yield different amounts of PCR product.

Annex IV: Theme 3, taskC.2.3: Analytical description of DNA quality

Appendix 1. Column and gradient specifications

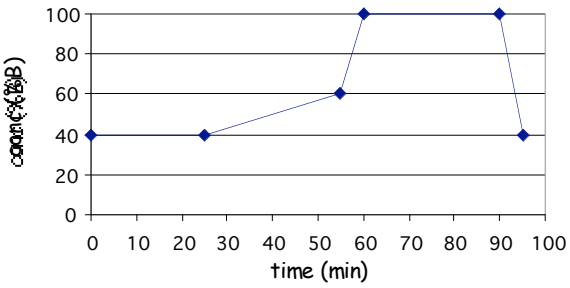
Appendix 1.1 Description of anion exchange chromatography columns

The first two columns, Waters protein pak DEAE 15 HR and Waters protein pak DEAE 8 HR, are composed of porous material to which DEAE groups (diethylaminoethyl) are covalently bound. These groups are weak anion exchangers that are only functional in a rather narrow pH range. “15 HR” and “8 HR” refer to the size of the porous beads. It is generally admitted that resolution will increase when bead size decreases.
The two others columns -Waters Gen Pak fax en Dionex DNA Pac PA100- are composed of non porous material and are able to endure much higher pressures. The Gen PAK Fax column is also characterised by the presence of DEAE groups whereas the DNA Pac PA100 column is characterised by the coating of non porous material with microbeads™ that are totally covered with quaternary amino groups. This allows functional separation in a very broad pH range with high resolution

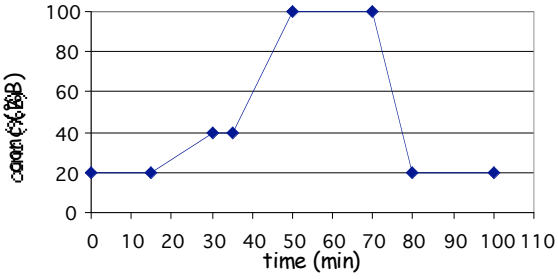
Appendix 1.2 Composition of elution gradients

Potassium Chloride gradients applied on DEAE 15 HR; DEAE HR8, Gen-Pak-Fax and DNA Pac PA-100
Buffer composition
buffer A: 25 mM Tris-HCl, 1 mM Na_2-EDTA, pH 8
buffer B: 25 mM Tris-HCl, 1 mM Na_2-EDTA, 1 M KCl, pH8

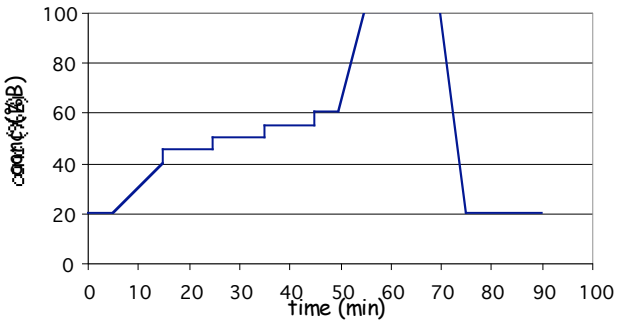
Gradient 4	Time (min)	Conc. B (%)
	0 -25	40
	25 - 55	40 - 60
	55 - 60	60 - 100
	60 - 90	100
	90 - 95	100 - 40



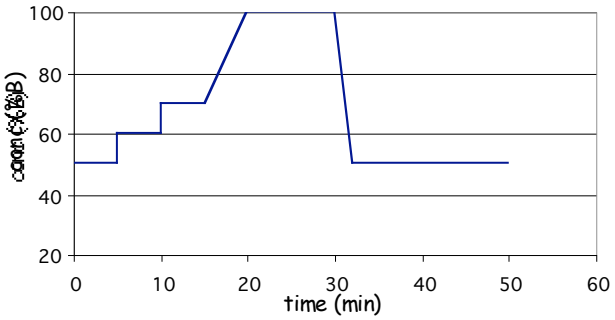
Gradient 8	Time (min)	Conc. B (%)
	0 - 15	20
	15 - 30	20 - 40
	30 - 35	40
	35 - 50	40 - 100
	50 - 70	100
	70 - 80	100 - 20
	80 - 100	20



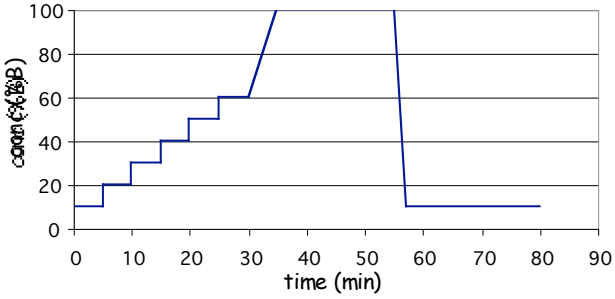
Gradient 11	Time (min)	Conc. B (%)
	0 - 5	20
	5 - 15	20 - 40
	15 -25	45
	25 - 35	50
	35 -45	55
	45 - 50	60
	50 - 55	60 - 100
	55 - 70	100
	70 - 75	100 - 20
	75 - 90	20



Gradient 14	Time (min)	Conc. B (%)
	0 -5	50
	5 - 10	60
	10 - 15	70
	15 - 20	70 - 100
	20 - 30	100
	30 - 32	100 - 50
	32 - 50	50



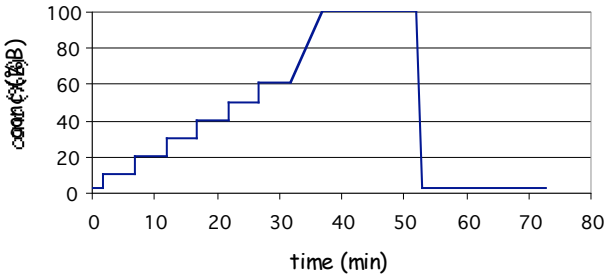
Gradient 17	Tijd (min)	Conc. B (%)
	0 - 5	10
	5 - 10	20
	10 - 15	30
	15 - 20	40
	20 - 25	50
	25 - 30	60
	30 - 35	60 - 100
	35 - 55	100
	55 - 57	100 - 10
	57 - 80	10



Sodium Perchlorate gradient applied on DNApac PA100

Composition buffer A: 25 mM Tris-HCl, 1 mM Na_2-EDTA, pH 8
buffer B: 25 mM Tris-HCl, 1 mM Na_2-EDTA, 0.375 M$NaClO_4$, pH8

Gradient 20	Time (min)	Conc. B (%)
	0 – 2	2
	2 - 7	10
	7 - 12	20
	12 - 17	30
	17 - 22	40
	22 - 27	50
	27 - 32	60
	32 - 37	60 - 100
	37 - 52	100
	52 - 53	100 - 2
	53 - 73	2



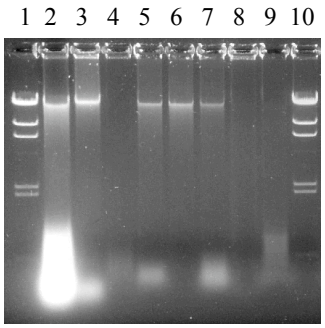
Appendix 2: Description of Primers and PCR conditions

All PCR programs were as follows:
1 x 5 min at 95°C
45 x 30 sec at 95°C
30 sec at Ta
30 sec at 72°C
1 x 6 min at 72°C

Table I: Primers

Target	Primer	Sequence	Length (bp)	Ta (°C)
Lectine	SL9	5'cat tac cta tga tgc ctc cac c 3'	178	55°C
	SL10	5' aag cac gtc atg cga ttc c 3'		
pLec plasmid	126F	5' tcc acc ccc atc cac att t 3'	81	58°C
	206R	5' ggc ata gaa ggt gaa gtt gaa gga 3'		

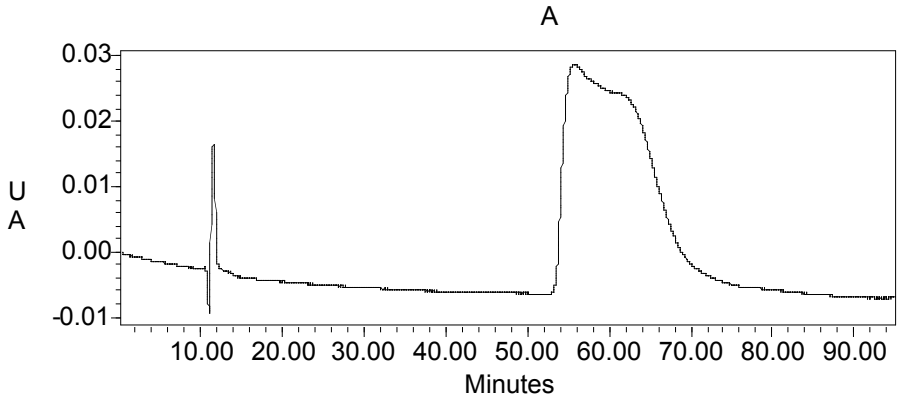
Appendix 3 : Results (Tables and Figures)



1 and 10. 500 ng / HindIII digest
2. gPC-02/01
3. gPCrnase-02/06
4. gCTAB-02/05
5. gHP-02/02
6. gHPrnase-02/07
7. gHP-02/08
8. gPFF-02/04
9. gTLSc-02/03

Electrophoresis gel : 0.8% agarose
25µl extract loaded

Figure 1: Gel electrophoresis in 0.8 % agarose of all tested soy DNA extracts. 25 µl of each extract is loaded on gel. The pLEC plasmid that was also analyzed in this work is not shown. Details on yields and concentrations are mentioned elsewhere.



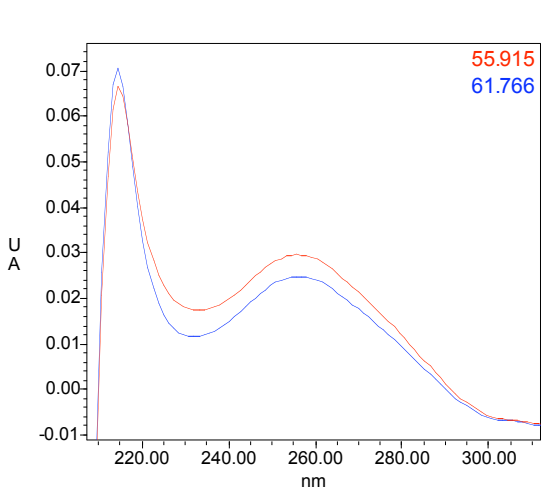


Figure 2: A: Chromatogram of 2,5 µg DNA-HindIII digest eluted on a DEAE 15 HR column with gradient 4 (appendix 1). **B** UV-spectrum of peaks at Rt = 55,9 min and Rt = 61,7 min.

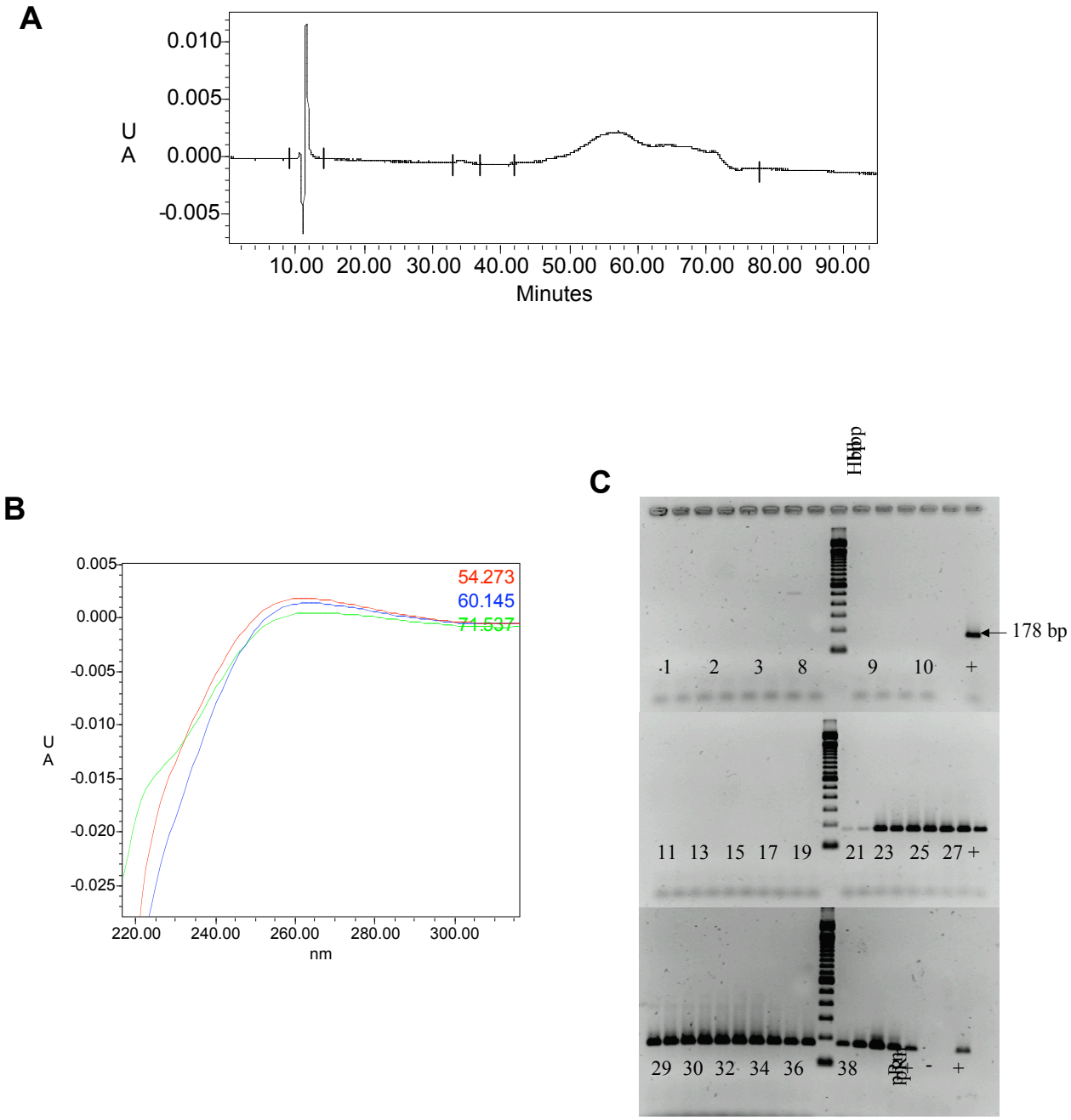


Figure 3: Analysis of HP-extract by anion exchange chromatography.
3A. Chromatogram of 2.2 µl HP-extract eluted on a DEAE 15 HR column with gradient 8 (see appendix 1). **3B** UV-spectrum analysis of peaks at Rt = 54,3 , 60,1 and 71,5 min. **3C** PCR amplification (appendix 3) of lectin gene (LE1) in HPLC fractions. F1 to F3 correspond to injection peak (Rt = 11,2 min); F8 to F11 correspond to peak with Rt = 33,8 min and F11 to F38 cover Rt = 47,9 min to Rt = 75,2. In a PCR analysis carried out on fraction starting from Rt = 75,2, positive signals were found up to Rt = 78,3 min

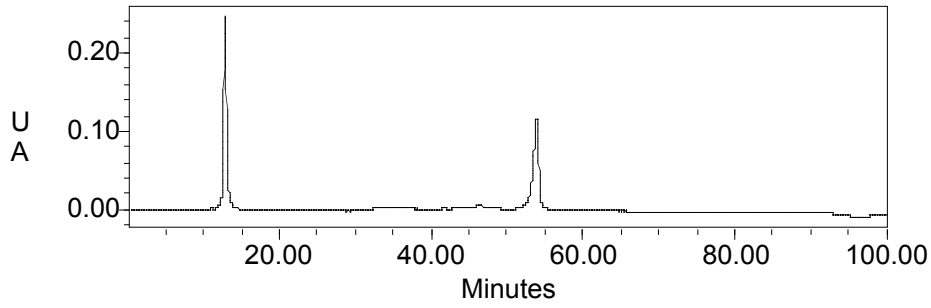


Figure 4 : Chromatogram representing an elution of 5 µg pLec plasmid on a DEAE 8 HR column with gradient 8 (details in appendix 1). UV-spectrum at Rt = 53,9 resemble those on Fig 2B. OD260/OD280 ratio at Rt = 53,9 min equals 1.99.

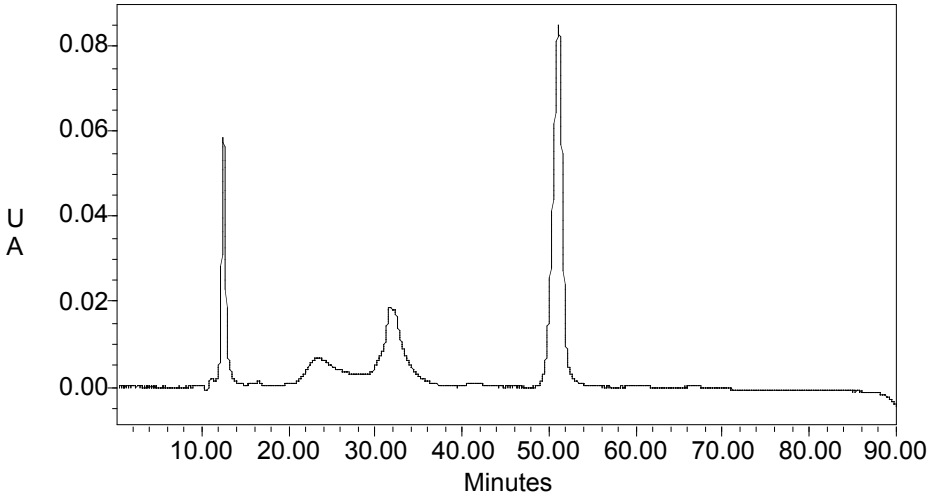


Figure 5 Chromatogram representing the elution of 5 µg pLec plasmid on a DEAE 8 HR column with gradient 11.

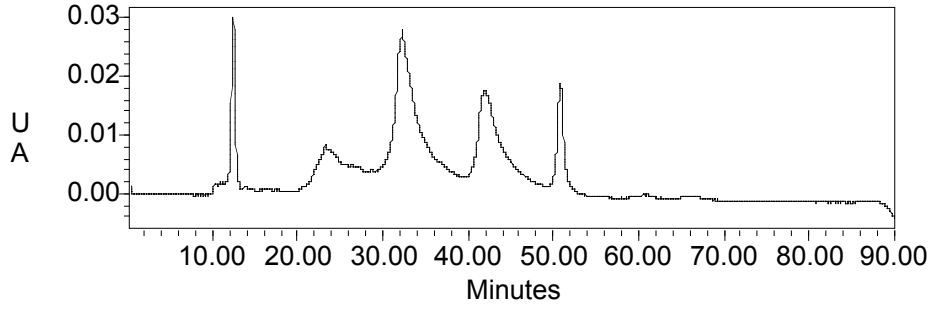


Figure 6 Elution of 6.4 µg PC-extract on DEAE 8HR column with gradient 11

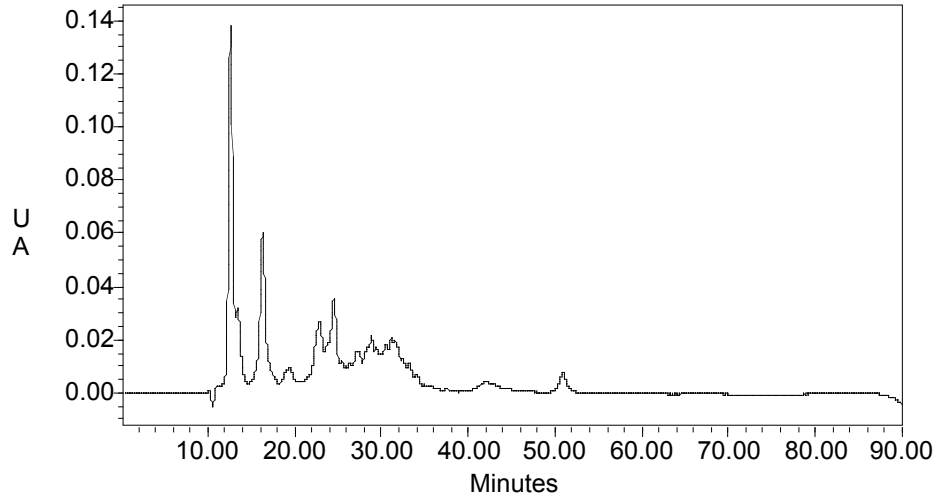
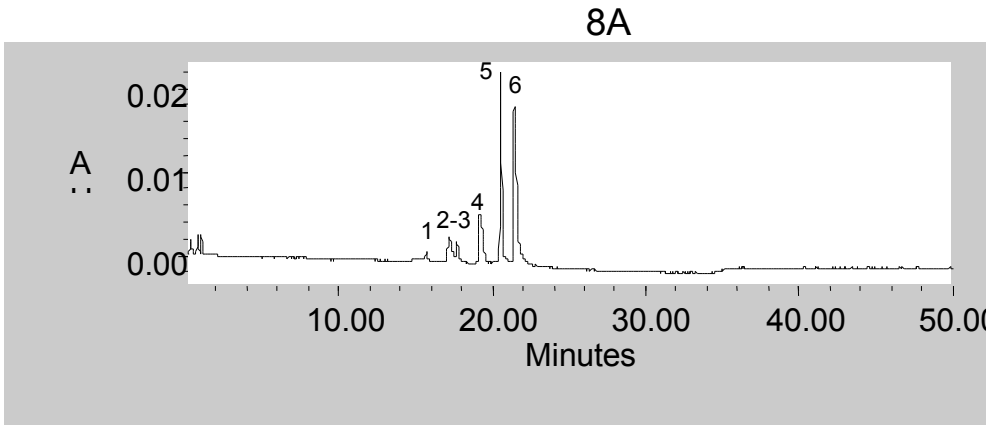


Figure 7 Elution of PC-extract with gradient 11 after a 10 min-RNase digestion at 37°C. Elution conditions are identical to those in Fig 6.



8B

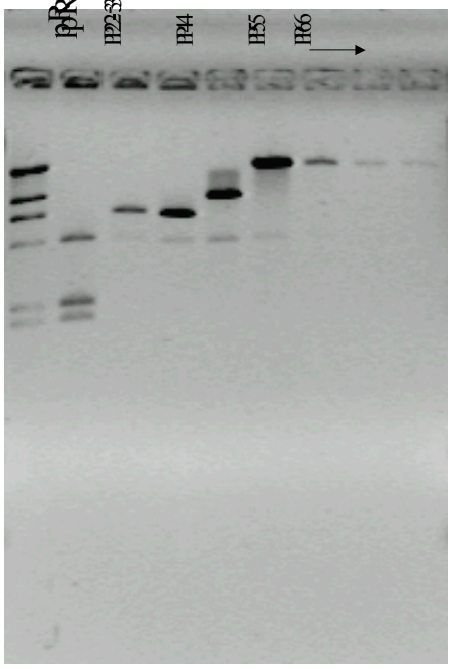
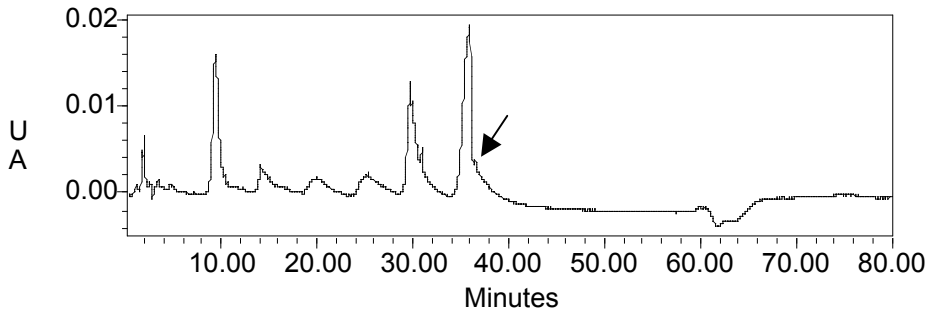
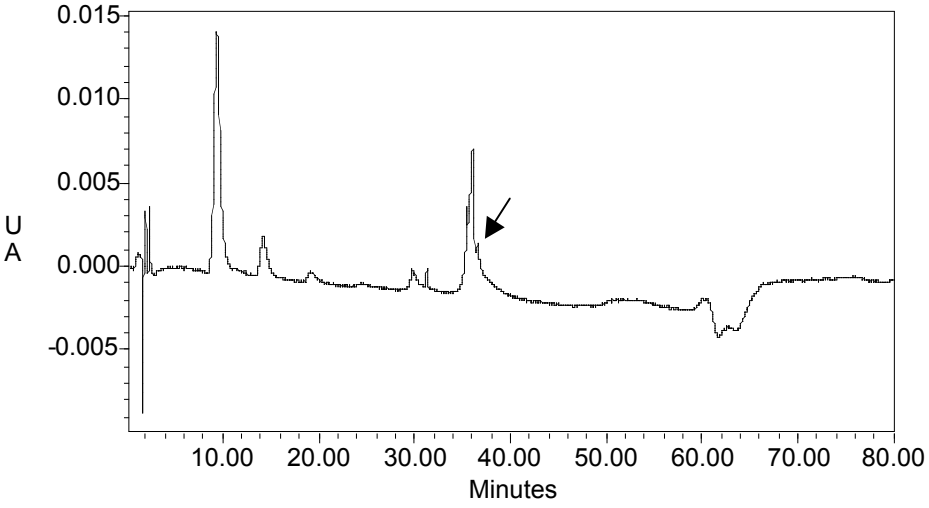


Figure 8 A Elution of 2,5 µg l / HindIII digest with gradient 14 (appendix2). **B** gel electrophoresis on 1% agarose of fractions collected from peaks 2 to 6 after concentration and desalting on microcon 30 (Millipore). One-ml fractions were reduced to 50 µl and 20 µl is loaded on gel. Abbreviations: pRn, lHindIII digest fraction prior to elution (preRun).

A



B



C

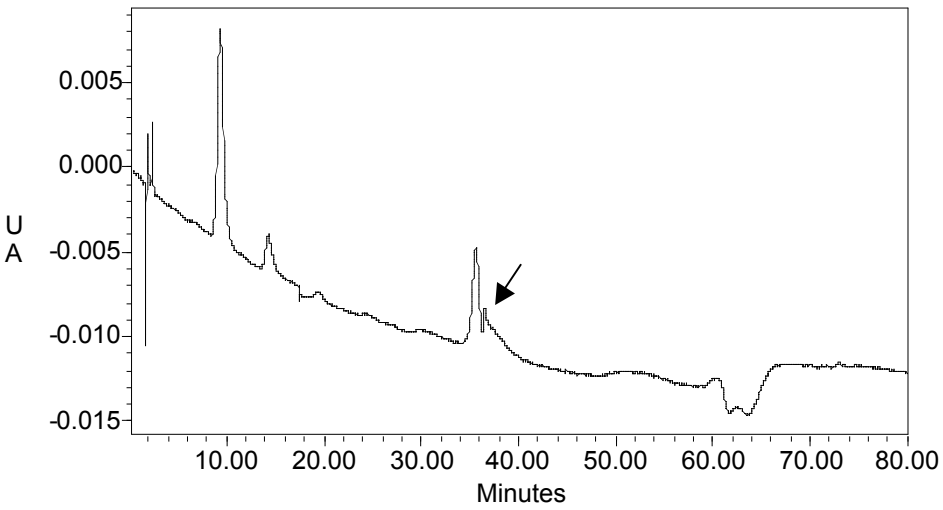
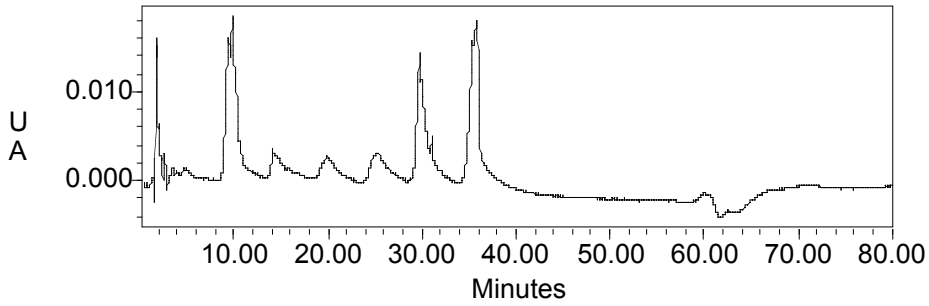
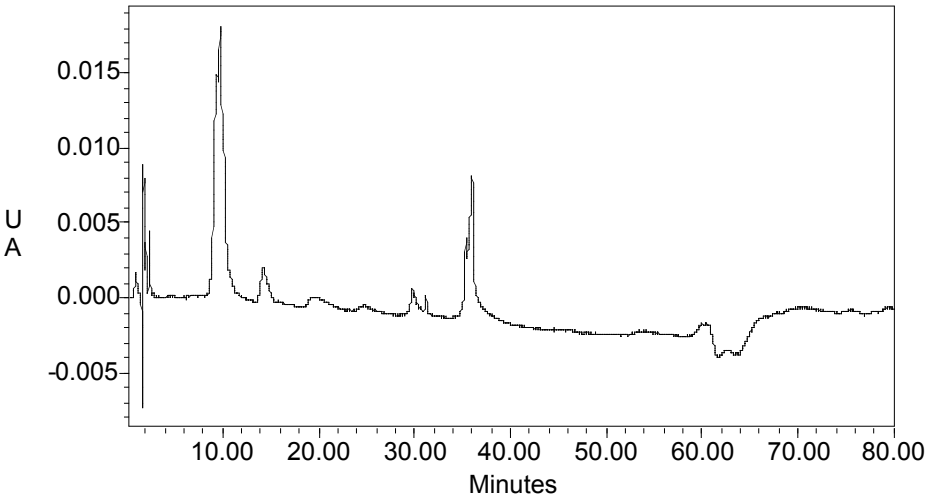


Figure 9 Elution of three genomic DNA extracts on a DNA Pac PA-100 column with gradient 17 (see appendix 1). Chromatogram of 6.4 µg PC-extract (**A**); 4,65 µg KF-extract (**B**) and 2,2 µg HP-extract (**C**). Black arrows refer to DNA peaks as suggested by PCR analysis and DNase digestions (see Fig.10)

A



B



C

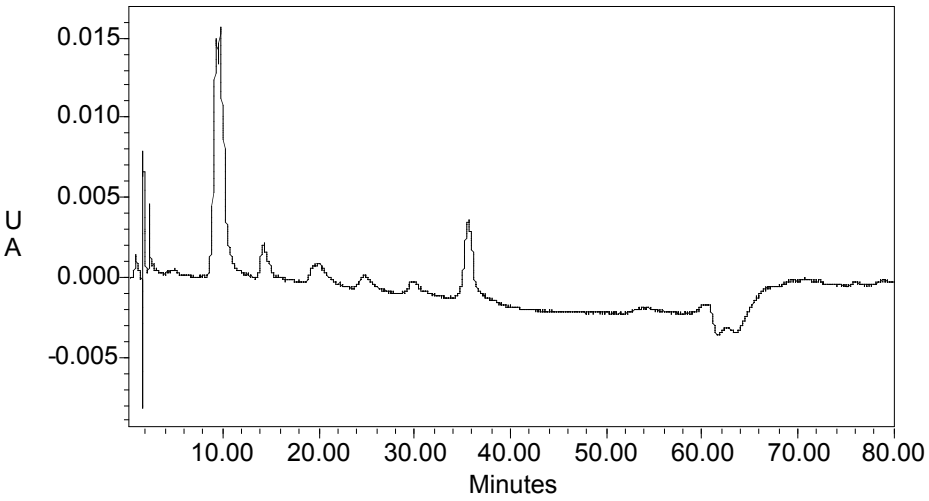


Figure 10 DNase treatment prior to elution of PC- (A), KF- (B) and HP- extract (C). Dnase incubation was carried out with 15 µl enzyme at 37°C for 40 min. Respectively 6.4 , 3,9 and 1,65 µg of PC-, KF- and HP-extracts were loaded onto the column. Elution was carried out with gradient 17.

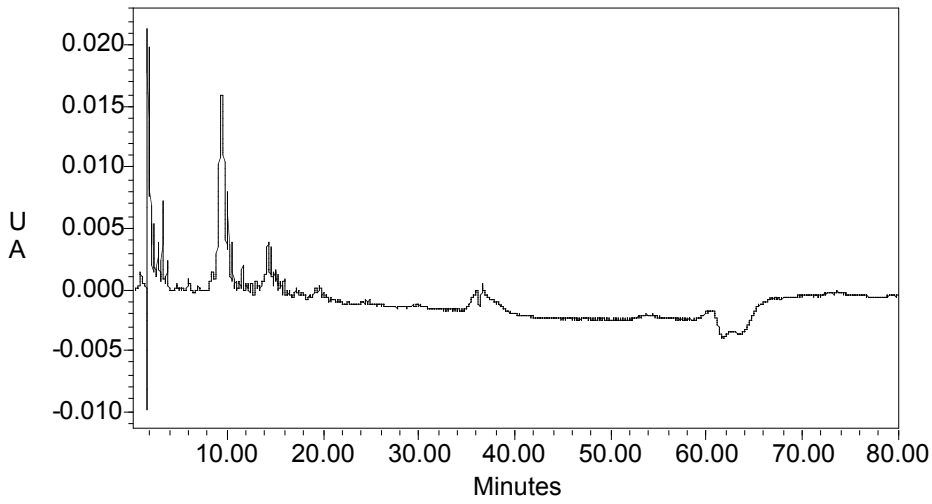


Figure 11 RNase digestion of 1,65 µg HP-extract with 5 µl enzyme for 30 min at 37°C prior to elution on a DNA Pac PA-100 column with gradient 17.

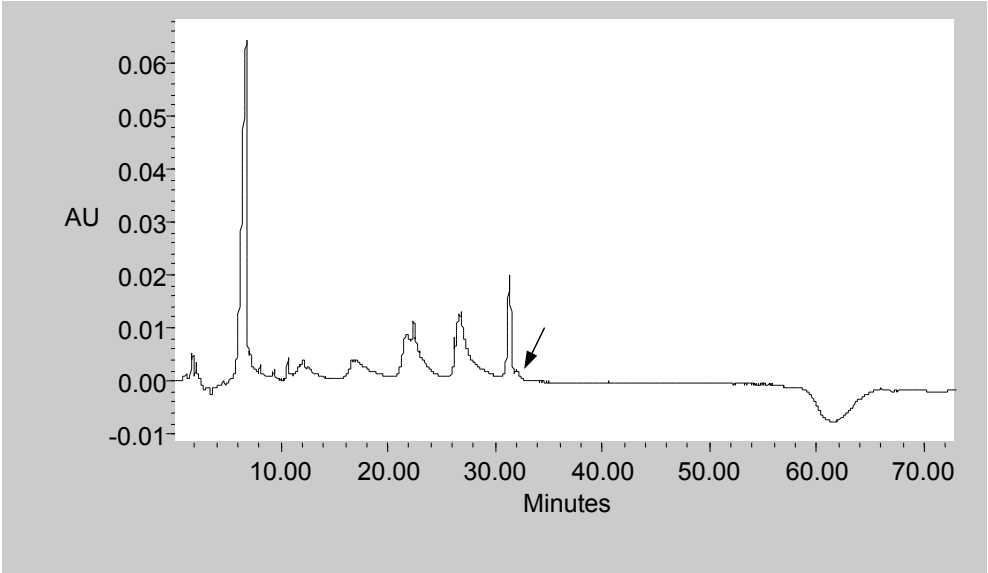


Figure 12 Elution of 6,4 µg PC-extract on DNA Pac PA-100 column with gradient 20 (appendix 1). Black arrow shows DNA peak.

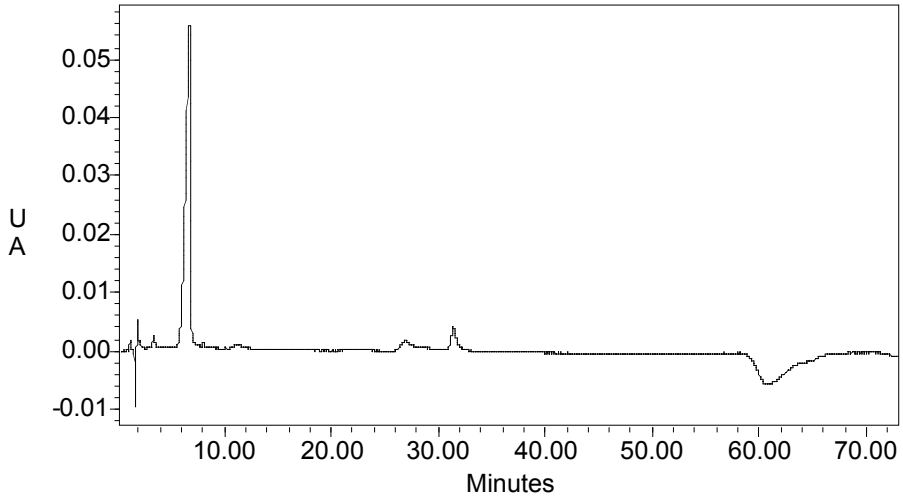
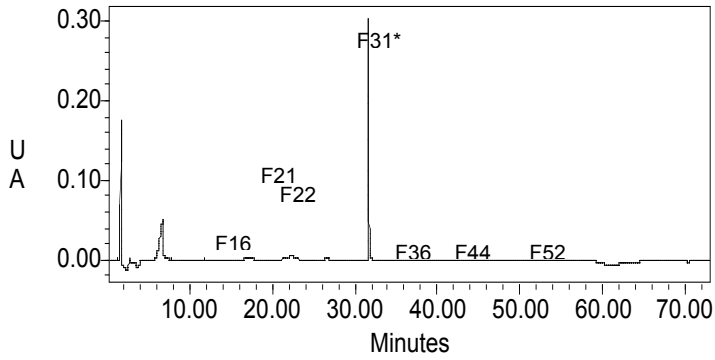


Figure 13 Elution of 1,45 µg CTAB extracts on DNA Pac PA-100 column with gradient 20. Peak at Rt = 31,8 min completely disappears upon DNase digestion (not shown)

A.



B.

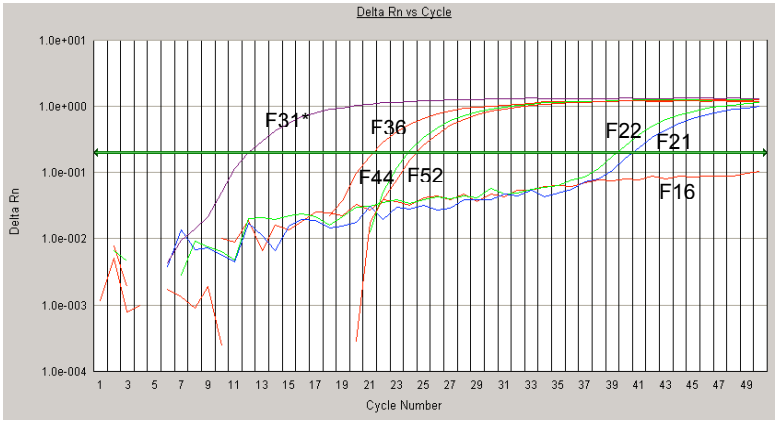


Figure 14: (A) pLec2 chromatogram (50 µl linear plasmid on DNA Pac100). (B) Real-time PCR results (promoter #5) on different fractions from pLec2 after HPLC purification on DNA Pac100

- **F16:** Fraction with no DNA
- **F21 and F22:** Fraction with probably traces of unrestricted plasmid
- **F31*:** Fraction with linear pLec2 plasmid fraction
- **F36, F44 and F52:** Fraction with residual plasmid (with visible « dilution effect »)