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# COMPARISON OF DNA EXTRACTION METHODS FOR GMO ANALYSIS OF FOOD PRODUCTS

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**Abstract.** We tested three methods for DNA isolation and purification based on different principles: a CTAB extraction protocol; the Maxwell™ 16 Tissue DNA Purification Kit (Promega) which facilitates an automated extraction using magnetic beads; and the High Pure GMO Sample Preparation Kit (Roche Diagnostics) based on affinity separation. Several types of food matrices derived from or containing soybean were used to test the performance of the selected methods. The experiment shows that the Maxwell™ 16 Tissue DNA Purification Kit is best suited for raw or low processed matrices such as seeds and flour. The other to methods are recommended for all types of samples. However. further optimization of the CTAB protocol is required in order to improve the quality and concentration of the extracted DNA.

## INTRODUCTION

The first commercial release of a GM crop took place in 1992. in China (Zhou *et al*.. 1995). Since then transgenic plants are spreading more rapidly than any other agricultural technology in history (Raney. 2006). Despite the potential benefits of this new technology. to improve the reliability and quality of the world food supply. public and scientific concerns have been raised about the environmental and food safety of GM crops (Nap *et al*.. 2003). These different views caused intense controversy.

In order to ensure transparency and to meet consumers' needs. EU legislation (e.g. Regulation EC No. 1829/2003 and Regulation EC No. 1830/2003) established new policies such as labeling. traceability and post-market monitoring of GMO derived food products.

Labeling is very important because it allows consumers to make an informed choice. Labels are applied based on results provided by accredited laboratories which apply molecular methods for DNA extraction. and qualitative and quantitative analysis. Qualitative analysis is based on PCR techniques. while quantitative measurement is archived using real-time PCR. technique considered to be the most powerful tool for quantitative nucleic acids analysis (Kubista *et al*.. 2006). These methods are also employed to first establish whether or not food products contain only EU authorized GMOs. In conclusion. DNA extraction is important for getting accurate and correct final results.

## MATERIAL AND METHOD

**Samples.** All samples used for this experiment are derived from soybean or contain it as ingredient. The samples are listed in Table 1.

**DNA extraction.** Genomic DNA was extracted using a CTAB-based protocol. the Maxwell™ 16 Tissue DNA Purification Kit (Promega) which uses magnetic beads and the High Pure GMO Sample Preparation Kit (Roche Diagnostics) based on affinity separation. For all samples and methods. extractions were repeated 3-4 times.

Table 1

Samples used to test the performance of extraction methods

| Sample | Type | Degree of processing |
|---|---|---|
| Roundup Ready® soybeans | Positive control | Raw |
| Certified Reference Material 410R SB-5 (Institute for Reference Materials and Measurements) | Positive control | Low processed |
| Flour | Unknown | Low processed |
| Textured soybeans | Unknown | Highly processed |
| Pate | Unknown | Highly processed |
| Cheese | Unknown | Highly processed |

The employed CTAB protocol was described by Somma (2004) and can be used for isolation of DNA from raw and processed plant matrices. The protocol comprises the following steps: 100 mg of homogenized sample are transferred into a sterile 1.5 ml microcentrifuge tube; 300 µl of sterile deionized water are added and the mix is homogenized with a loop; 500 µl of CTAB buffer (20 g/l CTAB. 1.4 M NaCl. 0.1 M Tris-HCl. 20 mM $Na_2EDTA$) are added and the mix is again homogenized; 20 µl Proteinase K (20 mg/ml) are added. the tube is shaken and incubated at 65 °C for 30-90 min; 20 µl RNase A (10 mg/ml) are added. the tube shaken and incubated at 65 °C for 5-10 min; the tube is centrifuged for 10 min at about 16000 g; the supernatant is transferred to a microcentrifuge tube containing 500 µl chloroform. the tube is shaken for 30 sec; the tube is then centrifuged for 10 min at 16000 g until phase separation occurs; 500 µl of upper layer are transferred into a new microcentrifuge tube containing 500 µl chloroform; the tube is shaken and then centrifuged for 5 min at 16000 g; the upper layer is transferred to a new microcentrifuge tube and 2 volumes of CTAB precipitation solution (5 g/l CTAB. 0.04 M NaCl) are added. the solution is mixed by pipetting; the tube is incubated for 60 min at room temperature and then centrifuged for 5 min at 16000 g; supernatant is discarded; the precipitate is dissolved in 350 µl NaCl (1.2 M) and 350 µl chloroform are added; the mix is shaken for 30 sec and then centrifuged for 10 min at 16000 g until phase separation occurs; upper layer is transferred to a new microcentrifuge tube; 0.6 volumes of isopropanol are added; the tube is shaken and centrifuged for 10 min at 16000 g; the supernatant is discarded and 500 µl of 70% ethanol solution are added; the tube is shaken carefully and then centrifuged for 10 min at 16000 g; the supernatant is discarded. the pellets are dried and DNA is re-dissolved in 100 µl of sterile deionized water.

The Promega kit we used is designed for automated DNA extraction from plant tissue samples using the Maxwell™ 16 platform (Promega). A pretreatment with the CelluACE™ XG System (Promega) is also recommended to improve the performance of the extraction. The procedure is designed for up to 50 mg of sample. CXG Buffer and CXG Enzyme Mix are added to each sample and then the tubes are incubated to 65 °C for 2 hours. After incubation. the whole mix is transferred in the Maxwell™ 16 DNA Purification Cartridge. The cartridge is then loaded into Maxwell™ instrument and the protocol is performed according to the specifications in the Technical Manual. At the end of the automated procedure. the DNA is eluted into 300 µl of buffer provided with the kit. Initially. 100 µl of CXG Buffer and 5 µl of CXG Enzyme Mix were used for the pretreatment of each sample. We also tested other volume combinations of CXG Buffer and CXG Enzyme Mix.

The third protocol used in this experiment was specially designed for isolation of DNA from raw material and food products of plant origin. The kit was used according to producer's instructions: 200 mg of homogenized sample is added in a 2 ml microcentrifuge tube; 1 ml of Extraction Buffer is added. the tube is vortexed for 30 sec and than incubated at 80 ºC for 30 min; during incubation the tube is mixed 2-3 times by inversion; the tube is centrifuged for 10 min at 12000 g and the supernatant is transferred into a new tube containing 400 µl of

Binding Buffer; 80 µl of Proteinase K are added and the tube is incubated at 72 °C for 10 min; 200 µl of isopropanol are added and 650 µl of the mixture are pipetted into the upper reservoir of a combined High Pure filter tube-collection tube assembly; the assembly is centrifuged for 1 min at 5000 g and the flow-through is discarded; the remaining mixture is applied to the assembly and the previous step is repeated; 450 µl of Wash Buffer are added to the upper reservoir and the assembly is centrifuged for 1 min at 5000 g; the flow-through is discarded and the washing step is repeated one more time; the tube is centrifuged for 10 s at maximum speed in order to remove residual Wash Buffer; the filter tube is inserted in a clean 1.5 ml reaction tube; 50 µl of pre-warmed (70 ºC) Elution Buffer is added onto the glass fiber fleece; incubate at 15-25 °C for 5 min and then centrifuge for 1 min at 5000 g; the 1.5 ml tube now contains the eluted DNA.

**DNA concentration. purity and fragmentation state.** Extracted DNA was first quantified using the BioPhotometer apparatus (Eppendorf AG). Concentrations (ng/µl) and $A_{260}/A_{280}$ readings were recorded for each sample.

Quality and quantity characteristics of the extracted DNA were further checked by electrophoresis on 1% (w/v) agarose gel (TAE buffer system) and ethidium bromide staining (0.5 µg/ml). The results were visualized on a BioSpectrum® AC Imaging System (UVP) using the Vision WorksLS software (UVP).

Table 2

Primers used for amplification of DNA extracts

| **Primers** | **Target** | **Size (bp)** | **Sequence 5'- 3'** | **Reference** |
|---|---|---|---|---|
| CP3<br>CP4 | *trnL* chloroplast intron | > 500 | GGG GAT AGA GGG ACT TGA AC<br>CGA AAT CGG TAG ACG CTA CG | Thion *et al*.. 2002 |
| GM03<br>GM04 | *Le1* gene | 118 | GCC CTC TAC TCC ACC CCC ATC C<br>GCC CAT CTG CAA GCC TTT TTG TG | Meyer *et al*.. 1996<br>Queeci and Mazzarra. 2004 |
| p35S-cf3<br>p35S-cr4 | 35S promoter | 123 | CCA CGT CTT CAA AGC AAG TGG<br>TCC TCT CCA AAT GAA ATG AAC TTC C | Lipp *et al*.. 2001<br>Queeci and Mazzarra. 2004 |
| CaMV1<br>CaMV2 | 35S promoter | 199 | GAA GGT GGC TCC TAC AAA TGC C<br>GTG GGA TTG TGC GTC ATC CC | Thion *et al*.. 2002 |
| HA-*nos* r<br>HA-*nos* f | *nos* terminator | 118 | GCA TGA CGT TAT TTA TGA GAT GGG<br>GAC ACC GCG CGC GAT AAT TTA TCC | Lipp *et al*.. 2001<br>Queeci and Mazzarra. 2004 |
| RR01<br>RR04 | GTS 40-3-2 transf. event | 356 | TGG CGC CCA AAG CTT GCA TGG C<br>CCC CAA GTT CCT AAA TCT TCA AGT | Studer *et al*.. 1998<br>Tengel *et al*.. 2001 |
| GMO7<br>GMO8 | GTS 40-3-2 transf. event | 169 | ATC CCA CTA TCC TTC GCA AGA<br>TGG GGT TTA TGG AAA TTG GAA | Meyer and Jaccaud. 1997<br>Querci and Mazzarra. 2004 |

**PCR.** The extracts were also tested for PCR using different primers specific for the chloroplast DNA. the soybean *lectin* gene *Le1*. the CaMV 35S promoter. the *nos* terminator and the GTS 40-3-2 transformation event (Table 2).

One PCR reaction contained 1X Green GoTaq® Reaction Buffer (Promega). 2.5 mM $MgCl_2$ (Promega). 0.2 mM of each dNTP (Promega). 0.5 µM of each primer. 0.03 U/µl of GoTaq® DNA Polymerase (Promega). 2µl of DNA solution with a concentration of less than 100 ng/µl and nuclease free water up to a final volume of 25 µl. The reactions were performed on a Palm Cycler™ thermalcycler (Corbett Research) using the amplification profile from Table 3.

PCR results were evaluated by electrophoresis (TAE buffer system) on 2% (w/v) agarose gel and ethidium bromide staining (0.5 µg/ml). To visualize the stained amplicons BioSpectrum® AC Imaging System (UVP) with the Vision WorksLS software (UVP) was employed.

Table 3

Amplification profile for PCR tests

| Step | Temperature (°C) | Time (seconds) | Repetition |
|---|---|---|---|
| Initial denaturation | 95 | 180 | 1 |
| Denaturation | 95 | 30 | 40X |
| Annealing | 60* or 63** | 30 | |
| Extension | 72 | 30 | |
| Final extension | 72 | 180 | 1 |

* For RR01/RR02 and GM07/GM08 primers.

** For CP3/CP4. GM03/GM04. p35S-cf3/p35S-cr4 and CaMV1/CaMV2 primers.

## RESULTS AND DISCUSSIONS

**DNA extraction. concentration. purity and fragmentation state.** Spectrophotometer and electrophoresis results indicated poor concentration and quality of extracts. However. the protocols performed much better in the case of seed and flour samples. compared to the highly processed ones (Table 4). No standard deviation was calculated here because samples were never extracted in more than two replicates.

Table 4

Concentration and purity of DNA extracts

| **Sample type** | **CTAB extraction. overall average of samples** | | **Maxwell™ 16 DNA Purification Kit. overall average of samples** | | **High Pure GMO Sample Preparation Kit. overall average of samples** | |
|---|---|---|---|---|---|---|
| | **Concentration (ng/µl)** | **$A_{260}/A_{280}$** | **Concentration (ng/µl)** | **$A_{260}/A_{280}$** | **Concentration (ng/µl)** | **$A_{260}/A_{280}$** |
| Soybeans | 104.3 | 1.95 | 87.1 | 1.24 | 1543.3 | 1.83 |
| CRM 410R SB 5 | 76.8 | 1.45 | 60.6 | 1.4 | 3035.5 | 2.04 |
| Flour | 90.1 | 1.32 | 75.4 | 1.35 | 1684.8 | 1.91 |
| Textured soybeans | 23.8 | 1.25 | 29.2 | 1.33 | 3444.0 | 1.97 |
| Pate | < 5 | - | < 5 | - | 36.2 | 1.24 |
| Cheese | < 5 | - | < 5 | - | 23.8 | 1.18 |

We recommend that DNA extraction with Maxwell™ 16 Tissue DNA Purification Kit should be performed on samples pretreated with a mixture of 250 µl of CXG Buffer and 25 µl of CXG Enzyme Mix. This volume prevents the complete absorption of the solution by the sample and ensures better homogenization.

Good results were obtained with the High Pure GMO Sample Preparation Kit. In this case concentrations were higher compared to the other extraction methods. and the purities were very good for seed. flour and textured soybean samples.

The electrophoresis indicated low concentrations of DNA for soybeans and flour. and even absence of DNA in case of other samples (Figure 1). For raw and low processed samples. extracts also held DNA of high molecular weight. DNA solutions obtained with the High Pure GMO Sample Preparation Kit had a high concentration of nucleic acid. as indicated by spectrophotometer readings. but most of the molecules were highly fragmented (Figure 1).

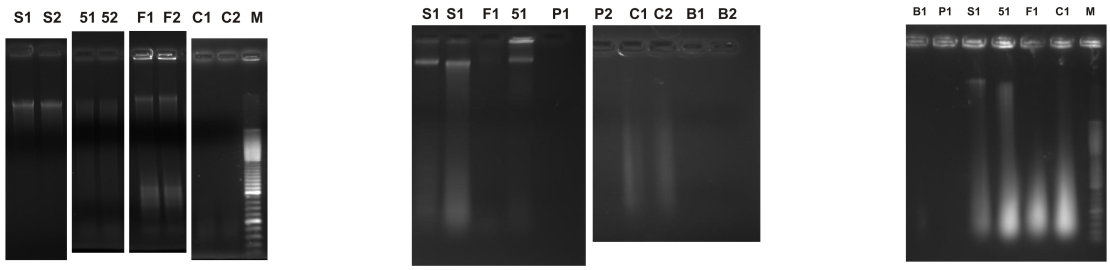


**Figure 1.** Gel electrophoresis of DNA extracts: Maxwell™ 16 Tissue DNA Purification Kit (left). CTAB-based protocol (middle). High Pure GMO Sample Preparation Kit (right). Samples: S - soybeans. 5 - CRM 410R SB 5. F - flour. P - pate. B- cheese. C - textured soybeans. M - marker. NTC - negative control.

**PCR** (Figures 2 and 3)**.** PCR tests gave goods results for all sample types when using plant and species specific primers. For GMO specific primers. only the positive controls and the flour unknown sample showed the expected amplicons. The pate. cheese and textured samples were negative. We concluded that in these samples the GM derived DNA was either absent or in quantities below the LOD.

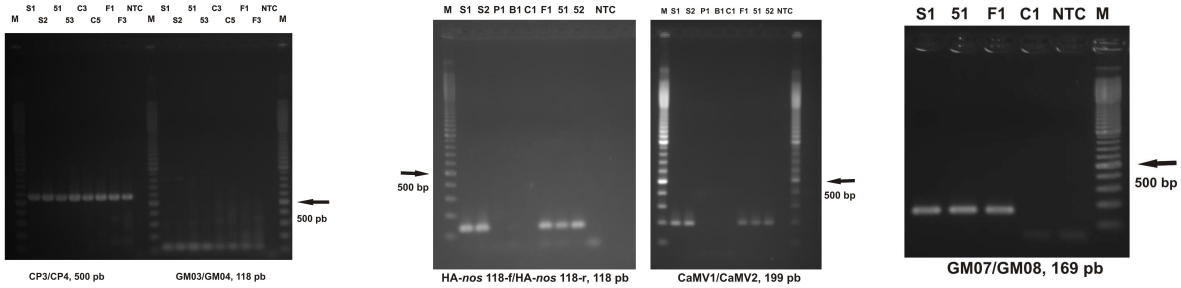


**Figure 2.** PCR results with the Maxwell™ 16 Tissue DNA Purification Kit. Samples: S - soybeans. 5 - CRM 410R SB 5. F - flour. P - pate. B- cheese. C - textured soybeans. M - marker. NTC - negative control.

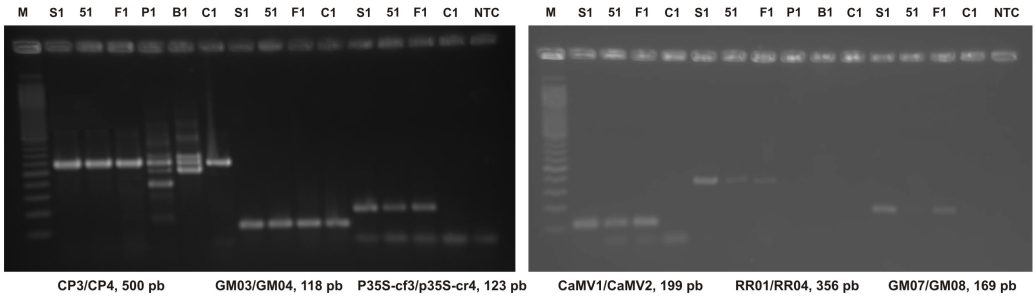


**Figure 3.** PCR results with the High Pure GMO Sample Preparation Kit. Samples: S - soybeans. 5 - CRM 410R SB 5. F - flour. P - pate. B- cheese. C - textured soybeans. M - marker. NTC - negative control.

## CONCLUSIONS

For validation of results. absence of PCR inhibitors should also be checked. This is possible in a real-time PCR amplification experiment using serial dilutions of the samples spiked with a defined copy number of a reference target sequence.

Only one of the analyzed samples was quantified so far by real-time PCR. using a Rotor-Gene™ 3000 instrument (Corbett Research) and Biogenics RoundUp™ Ready Soya QT Kit (Biotools). specially designed to be used with this apparatus. The sample was derived from CRM 410R SB 5. which has a 5% content of GM Roundup Ready® soybean. and was extracted with the Maxwell™ 16 Tissue DNA Purification Kit. We obtained a concentration of 5.31%. The value indicates a good performance of the integrated protocol. but analysis should be extended to all DNA extracts after optimization of protocols.

The tests showed that all methods are suited for further optimization. in-house validation and implementation in our laboratory as part of an integrated protocol for GMO analysis of foodstuffs. The manual CTAB-based method is the one needing most refinements. Another protocol. similar to this one. is available in SR EN ISO 21571:2005 and will also be tested.

We also concluded that the Maxwell™ 16 Tissue DNA Purification Kit is best suited for low processed matrices. while CTAB extraction and the High Pure GMO Sample Preparation Kit give good results for both categories.

Another aspect that we want to point out is the cost per analyzed sample. The Maxwell protocol is a little bit more expensive than the CTAB method. but compensates in terms of time required for processing and ease of use. Although the third kit probably delivers the best results it is also much more expensive that the other two methods.

## BIBLIOGRAPHY


1. Kubista. M.. J. M. Andrade. M. Bengtsson. A. Forootan. J. Jonák. K. Lind. R. Sindelka. R. Sjöback. B. Sjögreen. L. Strömbom. A. Ståhlberg and N. Zoric. 2006. The real-time polymerase chain reaction. *Molecular Aspects of Medicine. 27. 95-125.*
2. Lipp. M.. A. Bluth. F. Eyquem. L. Kruse. H. Schimmel. G. Van den Eede. E. Anklam. 2001. Validation of a method based on polymerase chain reaction for the detection of genetically modified organisms in various processed foodstuffs. *European Food Research Technology. 212. 497-504.*
3. Meyer. R.. F. Chardonnens. P. Hubner. J. Luthy. 1996. Polymerase chain reaction (PCR) in the quality and safety assurance of food: detection of soya in processed meat products. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A 203. 339-344.*
4. Meyer. R. and E. Jaccaud. 1997. Detection of genetically modified soya in processed food products: development and validation of a PCR assay for the specific detection of Glyphosate-Tolerant Soybeans. *Porceedings of the EURO FOOD CHEM IX Conference. Interlaken. Switzerland. Event No. 220. 1. 23-28.*
5. Nap. J.-P.. P. L. J. Metz. Marga Escaler. A. J. Conner. 2003. The release of genetically modified crops into the environment. Part I. Overview of current status and regulations. *The Plant Journal. 33. 1-18.*
6. Querci. Maddalena. M. Mazzara. 2004a. Characteristics of Roundup Ready soybean. MON810 maize. and Bt-176 maize In: *Querci. Maddalena. M. Jeremi. G. Van den Eede. The analysis of food samples for the presence of genetically modified organisms.* http://gmotraining.jrc.it/.
7. Raney. T.. 2006. Economic impact of transgenic crops in developing countries. *Current Opinion in Biotechnology. 17. 1-5.*
8. Somma. M.. 2004. Extraction and purification of DNA In: *Querci. Maddalena. M. Jeremi. G. Van den Eede. The analysis of food samples for the presence of genetically modified organisms.* http://gmotraining.jrc.it/.
9. SR EN ISO 21571:2005 Foodstuffs. Methods of analysis for the detection of genetically modified organisms and derived products. Nucleic acid extraction.
10. Studer. E.. C. Rhyner. J. Lüthy. P. Hübner. 1998. Quantitative competitive PCR for the detection of genetically modified soybean and maize. *Z. Lebensm. unTERS. Forsch.. 207. 207-213.*
11. Tengel. C.. P. Schusler. E. Setzke. J. Balles. M. Sprenger-Hausels. 2001. PCR-based detection of genetically modified soybean and maize in raw and highly processed foodstuffs. *BioTechniques. 31. 426-429.*
12. Thion. L.. Christine Vossent. Bettina Couderc. Monique Erard. Blandine Clemencon. 2002. Detection of genetically modified organisms in food by DNA extraction and PCR amplification. *Biochemistry and Molecular Biology Education. Vo.l 30. No. 1. 51-55.*
13. Zhou. R. H.. Z. C. Zhang. Q. Wu. R. X. Fang. K. Q. Mang. Y. C. Tian. G. L. Wang. 1995. Large-scale performance of transgenic tobacco plants resistant to both tobacco mosaic virus and cucumber mosaic virus. *In: Proceedings of the third International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Micro-Organisms (Jones. D. D.. Ed.). Oakland. CA: University of California. 49–55.*