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Developing a Rapid DNA-based Method for Detecting Soy Content in Foods

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Abstract: Food allergies are serious problem affecting more and more people, estimated that 1-2% of the population worldwide. In most cases, it requires a strict diet, which means that allergenic components should be eliminated from the diet. Soy contains several allergenic proteins that decrease the positive health effects of soy and cause potential allergenic symptoms in susceptible individuals. Accordance with the rules on the labelling of foodstuffs, due to the general labelling requirements for allergens, the presence of soy must be indicated on the labels of products containing soy, and the exemption must be checked regularly. In the frame of our research, our goal was to develop a fast, specific and highly sensitive test method to detect soy content. Firstly, we adapted and compared two DNA-based simple PCR methods by the means of using two different primer pairs (chloroplast AtpA gene-specific and LecI gene-specific) to detect DNA of Pannonia kincse, a Hungarian soybean variety. We found that AtpA-gene-specific primers were more effective/sensitive. Secondly a lateral flow soyDNA test was developed based on the selected chloroplast AtpA primerpair, recombinase polymerase amplification. We successfully confirmed the proper operation of the developed soy-specific DNA test by examining meat products with or without soy component. We could conclude that this developed test can be easily performed in situ, at any field (in non-laboratory conditions as shops, industry or restaurant), does not require complicated technical background and gives results already after 30-45 minutes. So this special qualitative test can be called as Field test.

Keywords: Soy Allergy, Field Test, Polymerase Chain Reaction, Isothermal Amplification, Recombinase Polymerase Amplification (RPA)

1. Introduction

The number of food allergies increases year by year, becoming a growing global problem. This may be due to an increased permeability of the intestinal epithelium, genetic predisposition (atopy), and difficulty in immune regulation. The type of triggered allergic reactions may be influenced by environmental factors such as contact with the antigen, type of food allergen or food processing, age and immunological immaturity [1-4].

The food allergy is a type I hypersensitivity reaction the pathomechanism of which is well known. Wide range of reaction symptoms include oral allergy syndrome (lip, tongue, palate irritation), skin symptoms (eczema), eye irritation, intestinal complaints (reflux, diarrhea), respiratory symptoms (nasal blowout, rhinitis, sore throat, asthma), cardiovascular diseases, and, in extreme cases, anaphylactic shock. Hypersensitivity response usually appear immediately after antigen exposure. To treat the symptoms, the food that causes allergic reactions should be left out of the diet [5].

Food allergies are caused by a wide variety of foodstuffs. More than 160 foods have been identified as allergens [6]. The most common food allergens that cause 90% of cases are labelled: cereals containing gluten, shellfish, eggs, fish, peanuts, soybeans, milk, nuts, celery, mustard, sesame, lupine, molluscs and products thereof [7]. Oral allergic syndrome can also be triggered by fresh vegetables and fruits, but they are not subject to labelling because of the lower frequency of allergies they cause.

Soy (*Glycine max*) belongs to the leguminous plants. It has been cultivated in Asian countries for thousands of years and consumed in different processed forms. Nowadays, the

largest quantities of soybeans are produced by the US, ahead of Brasilia, Argentina, China and India. Due to its high protein and oil content, it is widely used in the food industry. It has protein content of about 35-40% and oil content of about 20%, but these values are highly dependent on climate, geographical conditions and the variety of soy. The most soybeans are used in the meat industry. Various soyprotein isolates and concentrates help to replace meat proteins in different foodstuffs [8]. In addition, soy lecithin is used in the manufacture of confectionery products due to its excellent gelling and emulsifying abilities.

Soy proteins also have several beneficial properties, including the favorable amino acid composition, and components that help to reduce the cholesterol levels and the probability of hyperlipidemia and cardiovascular disease.

Other legumes as lupine and peanut, often used to replace soy in different foodstuffs may cause cross-allergenic symptoms. For this reason, the manufacturer must pay attention to the risk of such cross allergens. Multi-allergies should be taken into account mostly in childhood, so the simultaneous presence of soy, milk and egg can also be risky. The soybean allergens are 2S-albumins belonging to the group of storage proteins, trypsin inhibitors (Kunitz trypsin inhibitor, Bowman-Birk inhibitor), lipid transfer proteins, 11S-globulins (glycine), 7S-globulins (beta-conglycinin), and lectins. Soybean also contains non-protein-based anti-nutritive components that inhibit the absorption and utilization of useful nutrients [9].

According to the food labelling regulations, the presence of soy must be indicated on the label of the food. In addition, in the case of soy-free products, it is necessary to ensure that the food does not actually contain soy. Various protein-based methods, in particular immunological, electrophoretic and chromatographic methods [10-15] and DNA-based methods have already been developed to detect soy content [16, 17]. Our aim was to focus on the develelopment of a highly specific, rapid, and novel DNA-based method.

PCR primer pairs with higher detection limits were selected to develop a rapid isothermal -operate on constant temperature around 38°C - PCR method. We have choosen recombinase polymerase amplification (RPA) to achieve our goal to perform soy specific reaction requiring only a simple thermostat. RPA reagents are stable at room temperature and only strand displacing DNA polymerase and single strand binding recombinase required for the reaction. There are several efforts to design handheld-sized portable devices [18, 19]. RPA has mostly been reported in pathogen such as *Staphylococcus aureus* or *Lysteria monocytogenes* detection works [20].

2. Materials and Methods

2.1. Soybean Samples

Soybean samples were provided by Cereal Research Non-Profit Ltd., Szeged, Hungary. Optimization of the PCR reaction was carried out using the Hungarian soy cultivar „Pannónia kincse".

2.2. DNA Isolation and Primers

For DNA isolation, the Wizard® DNA purification system (Promega, Madison, Wisconsin, USA) and 100 g of homogenized soyflour were used. PCR reactions were performed on a Biometra TOne (Analytik Jena AG, Jena, Germany) gradient PCR instrument. Results were evaluated using a FlashGel™ electrophoresis device after PCR reactions.

So far the PCR method based on the detection of the so-called lectin gene was used to control the reliability of the soy-free products. Our research goal was to develop a highly sensitive, rapid DNA method for the detection of soy contaminants or soy ingredients in food. We developed the special qualitative test based on recombinase polymerase amplification which can be performed *in situ*, any field where it is necessary to determine soy content. Therefore, we adapted [22, 23] and compared two DNA-based methods using two different primer pairs (chloroplast AtpA gene-specific and LecI gene-specific), with special regard to their sensitivity (Table 1). The function of the Lec primer pair is based on the detection of the soybean lectin gene and the operation mechanism of the AtpA primer pair is based on the detection of the soybean's chloroplast AtpA gene.

Table 1. *Primers' parameters.*

Primer name	Sequence 5'→3'	Primer length	Specificity
AtpA-F	TGA TTC CCG AAC TTG ACC TC	20	Chloroplast AtpA gene
AtpA-R	AGT CGA GGA TTC GCT CGT TA	20	Chloroplast AtpA gene
Lec IF	CTT CTT TCT CGC ACC AAT	18	Lectin gene
Lec IR	CTC AAC AGC GAC GAC TTG	18	Lectin gene

During PCR optimization, we identified the optimal primer binding temperature, the appropriate DNA and primer concentration, and the optimal number of cycles by using both primer pairs. By specifying these optimal parameters, we were able to select the primary pair that could be used to detect the presence of soy even at very low concentrations. This optimization procedure substantiated the development of a rapid, sensitive, DNA-based so called Field-test method for the detection of soy content in food samples.

3. A rapid DNA-based Field Test

TwistAmp nfo kit was chosen from the different RPA applications. This kit utilize lateral flow strip as endpoint detection, so it operates as qualitative yes/no indicator [24]. The probe that use TwistAmp™ nfo kit (TwistDx Limited, Cambridge, United Kingdom) operates on 'sandwich'-assays principle. The reaction requires longer primers as usual, and

the melting temperature is not so important, as in the simple PCR method. One primer contains a biotin labelling at the 5' end. Beside the primer pairs, TwistAmp™ LF probe is required to the PCR reaction. Probe is a 46-56 bases-long oligonicleotide with a 5'- antigenic label (FAM) and contains a 3' polymerase extension blocking group. This probe also contained an abasic nucleotide analogue (tetrahydrofuran residue, THF), instead of a thymine nucleotide, which is about 30 base-long from the 5' end. In addition, it should be taken into account that the GC ratios (guanine-cytosine ratio) of the primers and the probe sequences are similar. Nfo enzyme [22] and recombinase polymerase were supplied in lyophilised form and could be solved and diluted to the final 50 µl reaction volume. Subsequently Milenia Genline Hybridetect strips (MileniaBiotec, Germany) has been used for the detection of successful RPA reaction, then immersed in the treated solution. This method works as a PCR combined with ELISA. 5 µl reaction volume was mixed with 100 µl strip-buffer, and then the strip was immersed into the solution. After 3-5 minutes, the control band at the upper labelled with RPA product appears visible (Figure 1). The biotin ligand – coupled to the surface of the strip - binds the RPA product. At that moment the FAM part of the RPA product is recognised by the anti-FAM antibody coupled with gold particles, so the Test line becomes. If no any band appears on the strip, amplification has been performed incorrectly. If there is only one band, the test sample does not contain soy in a detectable amount. If two bands appear, the sample contains a detectable amount of soy DNA. Primer-dimers do not give visible band, because FAM is on the probe (Figure 1).

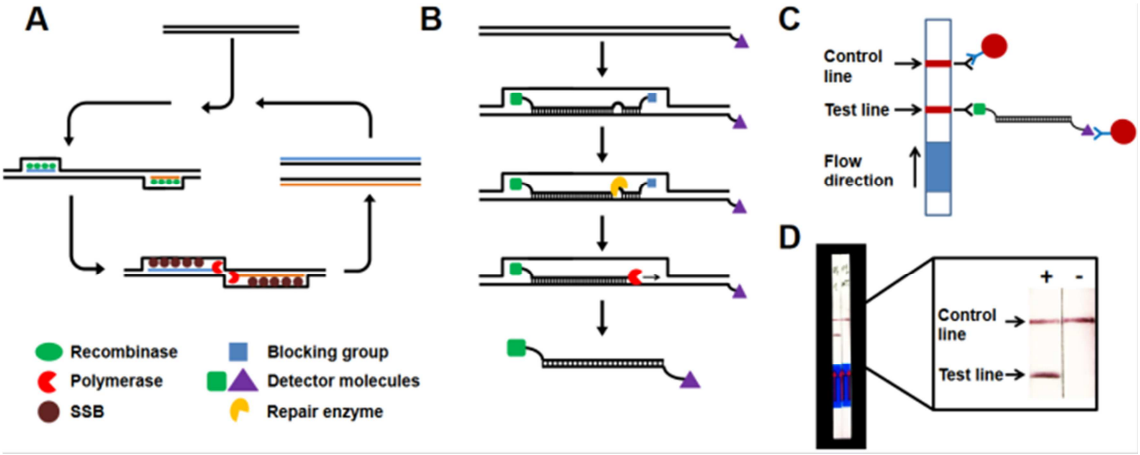

Figure 1. *Schematic representation of RFA and lateral flow detection principle [23].*

4. Results and Discussion

4.1. Optimizing the Polymerase Chain Reaction Parameters

The first parameter we analysed was the primer annealing temperature. In order to find the optimal temperature, we applied gradient PCR method, with adjusted annealing temperatures between 50 and 65°C in case of both primers (specific for AtpA gene and lectin gene). Monitoring the reaction by electrophoresis we found that the optimal annealing temperature was 58°C. For the further optimization, we complied a the PCR program which was summarized (vagy listed) in Table 2.

Table 2. *PCR program.*

Temperature	Time	Cycle number
95°C	10min	
95°C	30sec	
58°C	45sec	34x
72°C	30sec	
72°C	3min	
4°C	forever	

To determine the optimal DNA concentrate, twelwe different DNA concentrations were analysed between 10 and 120 µg/µl. According to the electrophoresis results the optimal DNA concentrate was 20 µg/µl. We found that it is optimal to use 1 µl 10 µM primer and 8.5 µl distilled water.

For the cycle number optimization, we analyzed 7 different cycle numbers: 25, 28, 31, 34, 37, 40, and 43. After the polymerase chain reactions the results were evaluated by gel electrophoresis. We found that 34 cycles and the reaction condition shown in Table 3. were optimal for the reactions using both AtpA and lectin gene specific reactions.

Table 3. *Optimal PCR parameters.*

Optimal PCR parameters	
Annealing temperature	58°C
DNA concentrate	20 ng/µl
Primer concentrate	10 µM/25 ml
Cycle number	34

The sensitivity of the methods had to be determined to compare the operation of the two primer pairs. We created dilution series for determination of detection limit. The initial concentration of our sample was 25 ng/µl, which contained around 23160550 copies from the target DNA sequence. So we made fivefold dilution series according to Table 4. and subsequently, soy-specific PCR reactions were performed using both primer pairs.

Table 4. *Correlation between sample dilution and DNA copy numbers.*

Sample number	Concentration ng/µl sample	Concentration ng/25 µl PCR solution	Copy number of target DNA
1	25	50	46323100
2	5	10	9264620
3	1	2	1852924
4	0.2	0.4	370584
5	0.04	0.08	74117
6	0.008	0.016	14823
7	0.0016	0.0032	2965
8	0.00032	0.00064	593
9	0.000064	0.000128	118
10	0.0000128	0.0000256	24
11	0.00000256	0.00000512	5
12	0.000000512	0.000001024	1

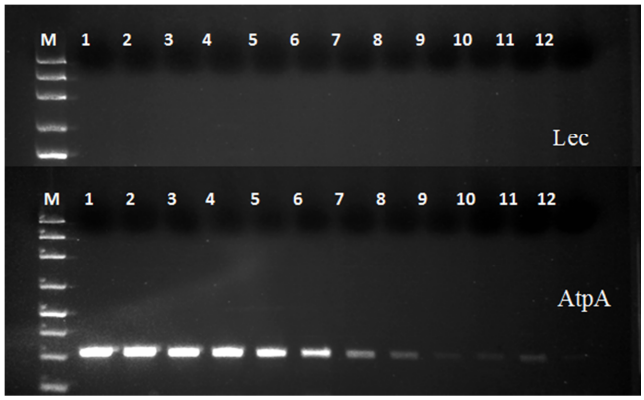

Figure 2. *Determination of the detection limit using Lec or AtpA primers. DNA marker (M): 100/200/300/500/800/1250/2000/4000 bp.*

The use of the AtpA primer is proved to be more sensitive than the use of Lec primer pair in testing the dilution series. This was also confirmed by testing different food samples (Figure 3). By using AtpA primers we detected samples containing soy in traces even at some soyfree labelled (1-4 sample) meat products, which proved the high sensitivity of this method.

Our results provided an appropriate basis for further research to develop rapid DNA-based field test for detection of soy-content in food samples.

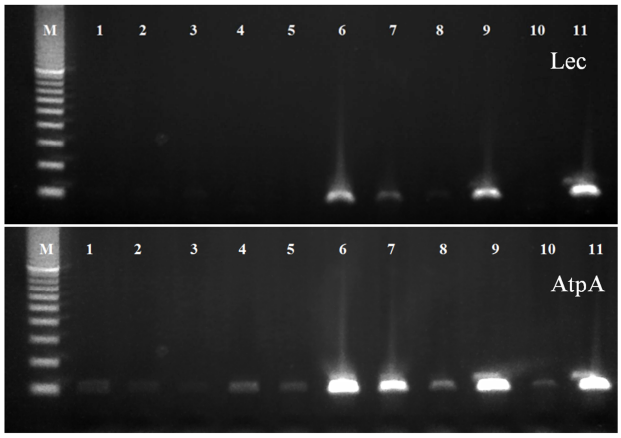

Figure 3. *Detection of soy content in different foods by PCR method with using Lec and AtpA primer pairs. M DNA marker 100/200/300/500/800/1250/2000/4000 bp; 1 turkey stew; 2 pig cold cuts; 3 turkey cold cuts; 4 beef cold cuts; 5 baked pig ham; 6 poultry cold cuts; 7 pork liver pate; 8 beef salami; 9 veal salami; 10 pig salami; 11. Pannónia kincse soy as a control sample.*

4.2. Optimizing the Rapid DNA-based Reaction

After selecting the AtpA primer for the field test - based on our results described above - we designed the primers and probe required for this method (Table 5).

Table 5. *Nucleotide sequences of primers and probe.*

Forward primer: Biotin-GAGAAGTTTCGAAGAAGGTGTTTTGTTAGA-3’
Reverse primer: 5’- ACCAAAATTGGCAATTTTGGTCAGATAACC -3’
Probe: FAM –CAAGTAGTCTTTGAAAGAAGACATATGATTCG(THF)GGCTTTCTTTTCTATCC –3’

For the optimization we used the DNA of soy cultivar “Pannónia kincse”. During the optimization procedure, the temperature (30-40°C) and time of the reaction (10-40 min) were changed between the values given in the protocol. The composition of the reaction solution was summarized in Table 6.

Table 6. *Composition of the reaction solution for soy-specific Field-test.*

Rehydration solution	
Primer A (10µM)	2.1 µl
Primer B (10µM)	2.1 µl
TwistAmp™ LF Probe (10µM)	0.6 µl
Rehydration buffer	29.5 µl
H_2O	12.2 µl

For each sample, we transferred 47.5 µl of the rehydration solution to the freeze-dried reaction pellet and then mixed by pipetting up and down until the pellet has been resuspended. After that we added 1 µl (50 ng/µl) DNA template to the rehydration solution, and then 2.5 µl Magnesium Acete (280 mM) to each sample. Accordance with the instruction of the TwistAmp nfo kit, the RPA reaction starts immediately after the Magnesium Acetate is added, even at room temperature. After the incubation reactions we verified the results using Milenia Genline Hybridetect strips. In the development of the soybean-specific field test, the most reliable and reproducible

results were obtained when the reaction lasted for 30 minutes and the temperature was 39°C.

4.3. Specificity

Various types of soybeans, as well as various plant and animal DNA samples were tested for specificity. As a result, we found that both plant and animal samples showed negative results using TwistAmp™ LF probe, but we received positive results for all the soy samples. It could be concluded that these kinds of primers and probe were exclusively specific to soy. (Table 7).

4.4. Detection Limit

For the measuring range analysis, we used 12 samples of fivefold diluted soy DNA as at the simple PCR reaction mentioned above (Table 4.) Based on our results the presence of soy in the sample can be also been detected in small copy number, we got weak signal at 1-24 DNA copy number too (Figure 4).

Table 7. *Demonstration of the specificity of our field test on studying different plant and animal samples.*

	Tested-samples	Number of samples	Number of positive results
Soy cultivars (domestic)	Pannónia kincse	50	50
	Bachia	20	20
	Aires	20	20
	Hilario	20	20
Plants	Peas	2	0
	Radish	2	0
	Potato	2	0
	Paprika	2	0
	Tomato	2	0
	Kohlrabi	2	0
	Blackberry	2	0
	Raspberry	2	0
	Eggplant	2	0
	Pear	2	0
	Pumpkin	2	0
	Spinach	2	0
	Almond	2	0
	Walnut	2	0
Animals	Carrot	2	0
	Celery	2	0
	Turnip	2	0
	Apple	2	0
	Pig	2	0
	Horned cattle	2	0
	Chicken	2	0
	Turkey	2	0

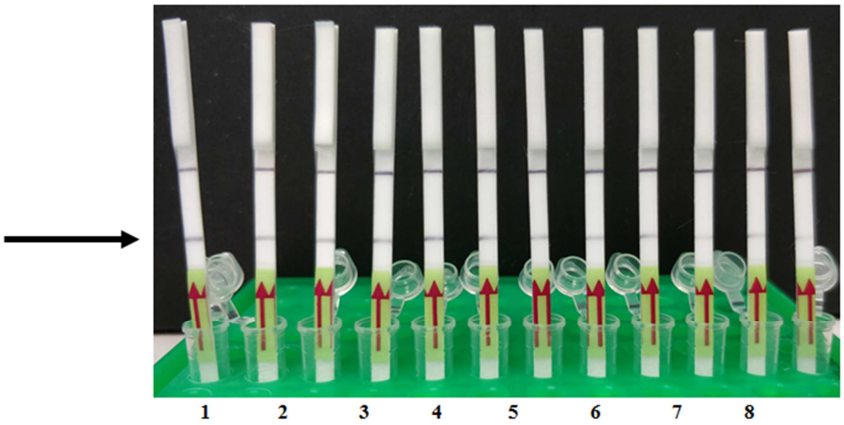

Figure 4. *Determining the sensitivity of the developed Field test.*

4.5. Analysis of Various Food Samples

We analysed meat products with different soy contents. After sample preparation and DNA extraction the analysis was carried out using TwistAmp™ LF probe with the optimized parameters. The method can also be applied in those cases when the tested sample contains reaction inhibiting components such as fat, spices as well. The method is sensitive and have positive results in high percentage in case of presumably „soy- free" labelled

products (Table 8).

Table 8. *Detection of soy content from different food samples.*

Sample type	Positive results (%)	Remark
Two-component model meat	100	10% soy content+90% pork
Raw sausage model-sample	100	10% soy content
Sausage model-sample	66	10% soy content
Turkey cold meat	66	„Soy-free”
Pork cold meat	66	„Soy-free”
Pork sausage	66	„Soy-free”
Turkey sausage	33	„Soy-free”
Oven roasted ham	33	„Soy-free”
Chicken cold meat	100	Contains soy
Pork liver pate	100	Contains soy
Beef salami	66	May contain traces of soy
Veal cold cuts	100	Contains soy
Cooked beef	33	May contain traces of soy

5. Conclusions

Detection of soy from foods is very important for allergic individuals to ensure their safety diets. We have successfully adapted two types of DNA-based methods (lectin gene and AtpA gene specific PCR methods) and optimized the polymerase chain reaction method for soybean detection in foods. Comparing the two primers, AtpA primers were found to be more sensitive, so the detection reaction was more effective. The explanation is that the lectin gene has one copy in a plant, while the AtpA gene is originated from chloroplast, and can be present in the eukaryotic organism in thousands of copies. To verify that “soy-free” products do not contain soy, PCR methods based primarily on the detection of the AtpA gene are highly offered to apply. Based on these primers we developed a rapid DNA-based method - TwistAmp™ nfo kit - to detect soy-content from food samples.

The developed RPA based field test is a sensitive and fast method for detecting soy ingredient in raw foods, food ingredients and food products, even if the sample matrice contains large amounts of fat, spices, that inhibit the conventional test methods. The field test can be used in non-laboratory conditions (shops, industry or restaurant) as the developed method is fast, easy to perform, does not require complicated technical background and the results are available after 30-45 minutes.

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