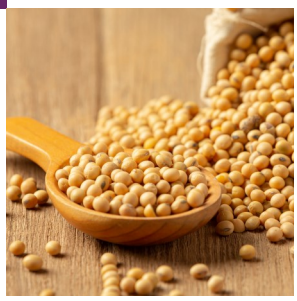
VALIDATION REPORT

Soy Free Test



Product code: E1310/E1330
Document no: E13[V3]
Date: 15 July 2024

PROGNOSIS BIOTECH

Soy Free Test Lateral Flow kit

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1. Introduction

Soybeans (mature seeds, raw) contain approx. 35 % proteins. The fraction of proteins in soy beans is very high. For this reason, soya is widely used as substitute for proteins from animal sources (e.g. in tofu, soya milk or soya yoghurt). Soya beans contain a high percentage of fat (approx. 20 %) and are used for the productions of oils and fats. Soy (*Glycine max*) belongs to the legumes. Many of these proteins are known for being allergenic, such as *Gly m1, Glycinin, Kunitz Trypsin Inhibitor* and *Gly m4* which is known to be cross reactive to birch pollen allergen *Bet v1*. For this reason, soy is one of the eight major food allergens. Consumption of soy might be harmful for people who are allergic to it. The allergen can be present as an ingredient or as a contamination in raw and cooked products. Consumption of soy-containing food from allergic people might cause a broad range of symptoms, such as hives, mild oral allergy or/and anaphylactic shock. Because of this, consumption of pecan or pecan containing food should strictly be avoided from allergic persons.

1.1 Regulations

According to the regulation (EU) No. 1169/2011 Annex II, soy is included in the list of allergens established by the European Food Safety Authority, and its presence must be indicated on the label. Similar regulations exist e.g. in the USA, Canada, Australia and New Zealand.

For this reason, it is of high importance to detect the presence of soy in food products labeled as soy-free. For more information on the allergens' regulations, visit our website: **www.prognosis-biotech.com.**

1.2 Principle of the method

The presence of soy in a sample is determined by the immunological detection of soy proteins. Antibodies specific to soy proteins are coated on the test line region (Test line) of the nitrocellulose membrane. During testing, antigens in the specimen react with the antibodies that are coated onto gold nanoparticles. The mixture migrates up the membrane to react with the antibodies immobilized on the membrane and generate a colored line in the test region T. The presence of the colored Test line indicates a positive result. In case of samples with a very high allergen concentration, the Test line fades or may not appear, giving us reduced or false negative result (hook effect phenomenon). For this purpose, a second line has been created (Hook line), whose intensity decreases as the amount of antigen increases and at very high concentrations it disappears either together with the Test line or before it. To serve as a procedural control, a colored line will always appear in the control region (Control line) if the test has been performed properly.

1.3. Kit Characteristics

See manual E1310/E1330 VN8

1.4 Method Protocol

Sample preparation

Solid Samples

- The sample must be collected according to established sampling techniques. Grind a representative sample (at least 5 g) to the particle size of fine instant coffee (50% passes through a 20 mesh screen).
- Weigh out a 0.5 g ground portion of the sample, add it into the prefilled sample tube and vortex it for 1 min (e.g. per 2-3 min). Alternatively, shake vigorously by hand. **The ratio of sample to extraction solvent is 1:10 (w/v).**
- Two (2) ml of the extract should be centrifuged at high speed for 2 min in reaction caps by using a microcentrifuge. Alternatively, let the sample settle down.
- Using a disposable pipette, transfer 3 drops from the supernatant to the empty extraction tube with the dropper tip.
- Dilute the sample **1:1** by adding 3 drops of the Matrix diluent (blue dropper tip) to the extraction tube with your supernatant.
- Close the extraction tube and shake well for a few seconds.

Liquid Samples

- Use 0.5 mL of the sample, add it into the prefilled sample tube and vortex it for 1 min. Follow the rest of the procedure exactly as described above for the solid samples. (You can skip using the microcentrifuge, unless your sample is a viscous liquid.)

Surfaces and swab sampling

- Mark out a swabbing area of approximately 10 x 10 cm.
- Moisten a swab by dipping into the extraction tube.
- Gather the sample with the swab by using a crosshatch technique. Move the swab horizontally, vertically, diagonally while rotating the tip. Repeat this starting from a different angle each time.
- After the sample collection, place the swab in the extraction tube, rotate the swab forcefully against the side of the tube for 1min. Best results are obtained when the sample is vigorously extracted in the solution. Remove the swab, squeezing the sides of the tube to extract as much liquid as possible. Shake vigorously for 1min by hand or on a vortex.
- Close the extraction tube. Add 3 drops into an empty extraction tube with the dropper tip. Dilute the sample **1:1** by adding 3 drops of the Matrix diluent (blue dropper tip).
- Close the extraction tube with the dropper tip.

NOTE 1: The extracted sample should have a pH value of 6.2 - 7.5. If the pH is less than 6.2 as for example happens on the silage samples, the pH should be neutralized using NaOH or HCl.

NOTE 2: In case of cloudy, thick samples, that do not allow the mixture to develop, an extra dilution 1:1 with the Extraction Buffer is required before transferring 3 drops to the reaction device. In this case, multiply the final soy proteins ppm result x 2.

NOTE 3: In case of meat samples divide your final ppm result by a factor of 0.39.

iii. Immunoassay Procedure and Interpretation of results (Total time: 5min)

- Add 3 drops in the circular window of the reaction device and allow the test to develop for 5 minutes.

Qualitative assessment

After 5 minutes, the test device can be visually read and interpreted as **1)Negative**, **2)Positive, 3)High Positive** or **4) Invalid**, according to a Visual result interpretation index

Quantitative assessment

After 5 minutes, place the reaction device inside the plastic holder in order to be scanned. Use the appropriate software to quantify results as soon as possible and no later than 1 minute after the end of analysis. The software will use a Lot specific curve to calculate the results. Refer to the Reader's manual for a detailed description of the quantification procedure.

2 Immunoassay Specifications

2.1 General Specifications

- Visual LOD of the method is 1.5 ppm (1.5mg/kg) soy proteins or 3.84 ppm soyflour in food samples and CIP solutions.
- Visual LOD of the method is 0.3125µg/100 cm^2 on working surfaces.

2.2 Assay Claims

- Samples showing negative results may contain soy below the limit of detection of the assay. This Lateral Flow kit does not claim that food is safe for consumption based upon a determination of soy content. Matrix effects may also affect the result of the method.
- The recovery/cross reactivity of the method might be affected when analyzing processed food (e.g. heat treatment, dehydration, etc.), because proteins may be altered or fragmented.
- The protein content and the protein composition may differ among various species of the same matrix. Therefore, different varieties may produce different results.
- LOD in CIP solutions refers to the final rinse water. The presence of cleaning agents and detergents may affect the result of the method.

3. Validation

3.1 Determination of the Limit of Detection LOD

The lowest detectable concentration of an analyte in a method is known as LOD. In this case, we check the concentration of a freshly prepared Soy Flour Extract , extracted and measured by **Allergen-Shield Soy** ELISA kit,A1848/ A1896. The LOD is the level at which 95% of the replicates are characterized as positive. The results of 20 replicates of 6 dilutions with Soy Flour Extract are shown at the table below.

Table 1. LOD of Soy flour in liquid extract

Concentration (ppm)	Positive Replicates	Visual Interpretation of results
50	20/20	Strong Positive
25	20/20	Strong positive
12.5	20/20	Positive
6.25	20/20	Positive
3.12	20/20	Positive
1.5	20/20	Positive
0.75	3/20	Negative

3.2 Specificity & Cross-Reactivity

- The target indicator protein of the antibody is soy glycinin, a major soy allergen selected based on its strong resistance to food processing and high abundance in the soybean.

For the determination of cross-reactivity 10 replicates of soy-free foods were tested. The samples were analyzed according to the experimental procedure described in Chapter 1.4.

Table 3. Cross-reactivities

No	Commodities	% Cross Reaction (N=10)	No	Commodities	% Cross Reaction (N=10)
1	Adzuki Bean	<LOD	47	Lima beans	<LOD
2	All purpose seasoning	<LOD	48	Lima beans flour	<LOD
3	Allspice	<LOD	49	Lupin	<LOD
4	Almond	<LOD	50	Macadamia nut	<LOD
5	Amaranth	<LOD	51	Millet	<LOD
6	Arrowroot	<LOD	52	Mung Bean	<LOD
7	Barley	<LOD	53	Mustard seed	<LOD
8	Beef	<LOD	54	Navy Beans	<LOD
9	Black Bean	<LOD	55	Non-Fat Milk Powder	<LOD
10	Black Pepper	<LOD	56	Nutmeg	<LOD
11	Brazil nut	<LOD	57	Oat	<LOD
12	Brown rice	<LOD	58	Oat flour	<LOD
13	Brown rice flour	<LOD	59	Paprika	<LOD
14	Buckwheat	<LOD	60	Peanut	0.0017%
15	Buckwheat flour	<LOD	61	Pecan nut	<LOD
16	Cashew	<LOD	62	Pine seed	<LOD
17	Cayenne Pepper	<LOD	63	Pinto beans	<LOD
18	Celery Seed	<LOD	64	Pinto beans flour	<LOD
19	Celery Flour	<LOD	65	Pistachio	<LOD
20	Cinnamon	<LOD	66	Poppy seed	<LOD
21	Chestnut	<LOD	67	Pork	<LOD
22	Chick peas	<LOD	68	Potato Starch	<LOD
23	Chick peas flour	<LOD	69	Pumpkin seed	<LOD
24	Chicken meat	<LOD	70	Quinoa	<LOD
25	Cocoa	<LOD	71	Rapeseed	<LOD
26	Coconut	<LOD	72	Rice	<LOD
27	Coffee	<LOD	73	Rye	<LOD
28	Corn	<LOD	74	Romano Beans	<LOD
29	Corn flour	<LOD	75	Sesame	<LOD
30	Crustacea	<LOD	76	Soy	100%
31	Cumin	<LOD	77	Soya flour	100%
32	Fava Bean	<LOD	78	Soy milk	100%
33	Flaxseed	<LOD	79	Sorghum	<LOD
34	Garbanzo Bean	0.0048%	80	Spelt	<LOD
35	Garfava Flour	0.0025%	81	Sunflower seed	<LOD
36	Green Bean Flour	<LOD	82	Sweet Rice	<LOD
37	Green Peas	<LOD	83	Tapioca	<LOD
38	Green Peas Flour	<LOD	84	Turkey meat	<LOD
39	Green Peas Protein	<LOD	85	Turmeric	<LOD
40	Hazelnut	<LOD	86	Walnut	<LOD
41	Hazelnut flour	<LOD	87	Wheat	<LOD
42	Kidney beans	<LOD	88	Wheat flour	<LOD
43	Kidney beans flour	<LOD	89	White beans	<LOD
44	Lecithin	<LOD	90	White beans flour	<LOD
45	Lentils	0.0010%	91	Whole Egg Powder	<LOD
46	Lentils flour	0.0008%			

3.3 Matrix Study

Spike Protocol

In this study we want to check the capability of our product to detect soy glycinin in different matrices and to confirm that the LOD of our method is 1.5ppm, as we described earlier in 3.1. All the samples were spiked with a freshly prepared Soy Flour Extract using ProGnosis Biotech Spiking Protocol. More particularly, all spike experiments were carried out by spiking the individual pre-weighed test portion with a concentration adjusted solution to maintain the spiking volume at 100 µL. The spike solution was prepared from a freshly prepared Soy Flour Extract. Soy-free samples were spiked using the above prepared stock solution with a positive displacement Hamilton syringe and were left open in a fume hood for approximately 30 minutes to allow the solvent to evaporate prior extraction. For the surfaces, we chose plastic, stainless steel and synthetic (working bench) as the material. . We cleaned a representative piece and we marked out 10 swabbing areas of approximately 10 x 10 cm. Each of these areas were spiked at 100µl with the stock solution and allowed to dry for 24 hours before sample collection. The resulting in-house spiked matrices and surfaces were extracted and analyzed according to the manual E1310/E1330 VN8 chapter 8.

i. Soy Free edible materials (Food)

For the first Matrix Study, 3 representative soy free matrices were spiked with the soy flour stock solution at 30ppm. Then, dilutions were made with Extraction buffer and, at each level, we used 10 replicates to check the efficiency of our product. Before we started, we confirmed that the samples did not contain soy and that the spike level was right by using our ELISA kit Allergen-Shield Soy, A1848/A1896.

Table 3. Matrix study of Soy Free Test on food

Matrix	Dilution	Number of Reaction Device (N=10)										Average
		1	2	3	4	5	6	7	8	9	10	
Instant soup powder	30ppm	+	+	+	+	+	+	+	+	+	+	10/10
	1/5 (6ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/20 (1.5ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/30 (1ppm)	+	-	+	-	-	-	-	-	-	-	02/10
Infant formula	30ppm	+	+	+	+	+	+	+	+	+	+	10/10
	1/5 (6ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/20 (1.5ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/30 (1ppm)	+	-	-	-	-	-	-	-	-	-	01/10
Cooked biscuit	30ppm	+	+	+	+	+	+	+	+	+	+	10/10
	1/5 (6ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/20 (1.5ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/30 (1ppm)	-	-	-	+	-	-	-	-	-	-	01/10

ii. Clean in Place Rinses (CIP SOLUTIONS)

For this study, 2 representative CIP solutions and Deionized water were spiked with the soy flour stock solution at 30ppm. Then, dilutions were made with Extraction buffer and, at each level, we used 10 replicates to check the efficiency of our product. Before we started, we confirmed that the CIPs did not contain soy and that the spike level was right by using our ELISA kit Allergen-Shield Soy, A1848/A1896.

Table 4. Matrix study of Soy Free Test on CIP solutions

Matrix	Dilution	Number of Reaction Device (N=10)										Average
		1	2	3	4	5	6	7	8	9	10	
Aciplus foam 1%	30ppm	+	+	+	+	+	+	+	+	+	+	10/10
	1/5 (6ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/20 (1.5ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/30 (1ppm)	-	+	+	-	-	-	-	-	-	-	02/10
Divosan VT5 0.5%	30ppm	+	+	+	+	+	+	+	+	+	+	10/10
	1/5 (6ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/20 (1.5ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/30 (1ppm)	-	-	-	-	-	+	-	-	-	-	01/10
Deionized water	30ppm	+	+	+	+	+	+	+	+	+	+	10/10
	1/5 (6ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/20 (1.5ppm)	+	+	+	+	+	+	+	+	+	+	10/10
	1/30 (1ppm)	-	-	-	+	-	-	-	+	-	-	02/10

iii. Surfaces-Swab Technique

For the surfaces, we chose plastic, stainless steel and synthetic (working bench) as the material. We cleaned a representative piece and we marked out 10 swabbing areas of approximately 10 x 10 cm. Each of these areas were spiked at 100µl with the stock solution and allowed to dry for 24 hours before sample collection. For each surface, the following replicate test portions were prepared: 10 at a soy level of 1.25µg/100 cm^2, 10 at a level of 0.625µg/100 cm^2,10 at a level of 0.3125µg/100 cm^2, and 10 at a level of 0.1562µg/100 cm^2. The result of this study is that the LOD of our product, for environmental swabs, is 0.3125µg/100 cm^2.

Table 5. Matrix study of Soy Free Test on surfaces

Surface	Spike level	Number of Reaction Device (N=10)										Average
		1	2	3	4	5	6	7	8	9	10	
Stainless steel	1.25µg/100 cm^2	+	+	+	+	+	+	+	+	+	+	10/10
	0.625µg/100 cm^2	+	+	+	+	+	+	+	+	+	+	10/10
	0.3125µg/100 cm^2	+	+	+	+	+	+	+	+	+	+	10/10
	0,1562µg/100 cm^2	-	-	-	-	-	+	-	-	-	+	02/10
Plastic	1.25µg/100 cm^2	+	+	+	+	+	+	+	+	+	+	10/10
	0.625µg/100 cm^2	+	+	+	+	+	+	+	+	+	+	10/10
	0.3125µg/100 cm^2	+	+	+	+	+	+	+	+	+	+	10/10
	0,1562µg/100 cm^2	+	-	-	-	-	-	-	-	+	-	02/10
Synthetic	1.25µg/100 cm^2	+	+	+	+	+	+	+	+	+	+	10/10
	0.625µg/100 cm^2	+	+	+	+	+	+	+	+	+	+	10/10
	0.3125µg/100 cm^2	+	+	+	+	+	+	+	+	+	+	10/10
	0,1562µg/100 cm^2	-	-	-	+	-	-	-	-	-	-	01/10

3.4 Performance Evaluation with Reference Materials

To evaluate the efficiency of Soy Free Test, we chose a wide range of reference materials that had been treated from FAPAS, an accredited proficiency testing provider. All samples were accompanied by certificates that confirm their assigned value. For each sample, 10 replicates were performed.

Table 6. Evaluation of Soy Free Test with samples prepared by FAPAS

Reference material	Assigned value (mg/kg) according to R-Biopharm Ridascreen Fast Soya	Range for |z|≤2	Result (N=10)	Average
FAPAS Wheat Flour	25.6	12.8 - 38.4	Positive	10/10
FAPAS Wheat Flour	NOT DETECTED	-	<LOD	10/10
FAPAS Wheat Flour	30.5	15.2 - 45.7	Positive	10/10
FAPAS Wheat Flour	NOT DETECTED	-	<LOD	10/10
FAPAS Wheat Flour	21.7	10.8 - 32.5	Positive	10/10
FAPAS Wheat Flour	5.61	2.81 - 8.42	Positive	10/10

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