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CAT.NUMBER: A1548/A1596

Allergen Shield Almond

ELISA TEST | In vitro analysis

for the quantitative determination of almond residues in food

ProGnosis Biotech S.A. is ISO 9001:2015 certified by TÜV Hellas (TÜV NORD).

Use only the current version of Product Data Sheet enclosed with the kit.

Allergen-Shield Almond, A1548/A1596, is an immunoassay method that determines almond residues in food products labeled as almond-free. The ELISA kit contains all reagents required for the immunoassay method. The ELISA test is adequate for 96/48 definitions (standards are included). A spectrophotometer for microtiter ELISA plate is required.

Matrices:

Beverages, Cake, Cereals, Chocolate, Cookies, Crackers, Ice cream and Spices

- Sample preparation: extraction
- Test time (incubation time after samples and reagents preparation): 30 min
- Standard curve range: 0 - 25 ppm
- Shelf life: 12 months
- Storage: 2-8^{o}C

Specifications

- The LOD of the method is 0.25 ppm almond or 0.053 ppm almond protein.
- The LOQ of the method is 1.0 ppm almond or 0.21 ppm almond protein.
- The recovery of spiked extractions-matrices was 90.1% (CV = 6.8%).
- IC50 = 4.0 - 9.0 ppm.
- Each standards duplicates mean CV ≤ 6%.
- Coefficient of Variation (CV) of result at 5 ppm = 5.8% (n=16).
- Coefficient of Variation (CV) of result at 15 ppm = 5.9% (n=16).
- The target indicator protein of the antibody is a major almond allergen and was selected based on its strong resistance to food processing and high abundance in the nut meat.
- The assay exhibits strong reactivity to almond (*Prunus dulcis*) and to a lesser extent mahalab (0.0546%), a spice prepared from the pit of cherry (*Prunus avium*). Cashew (0.0316%), sunflower seed (0.0065%), and oat (0.0012%) were minimally reactive in the assay.
- At concentrations as high as 100,000 ppm, no cross-reactivity was observed in the other matrices (including tree nuts, legumes, seeds, flour, meats and spices).

1. Description

Allergen-Shield Almond is an sandwich enzyme-linked immunosorbent assay (ELISA) test for the detection of almond protein residues in food products labeled as almond-free.

2. General Information

Almonds (*Prunus dulcis*) belong to the botanical family *Rosaceae*. Consumption of almond 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 almond-containing food from allergic people might cause a broad range of symptoms, such as hives, mild oral allergy or/and anaphylactic shock. According to the regulation (EU) No. 1169/2011 Annex II, almond 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.

3. Principle of the method

The presence of almond in a sample is determined by the immunological detection of almond proteins. The wells of the microtiter strips are coated with specific antibodies against almond proteins. The standard solutions and the solutions of the samples are added and if a specimen contains almond proteins, the latter will bind to the immobilized antibodies. All of the unbound almond proteins will be removed by washing. Then, the detection solution is added (peroxidase-conjugated antibody against almond proteins) and binds to almond proteins. Any unbound molecule of the detection solution will be removed by washing. A chromogen substrate is then added to the wells resulting in the progressive development of a blue colored complex with the detection antibody. The color development is then stopped by the addition of acid turning the resultant final product yellow. The measurement is made photometrically at 450 nm and the intensity of the produced colored complex is directly proportional to the concentration of almond proteins present in the samples and the standard solutions.

4. Reagents Provided

Allergen-Shield Almond ELISA kit contains sufficient reagents and materials for 48/96 measurements (including standard tests).

Reagents (Store at 2-8ºC)	Quantity for 48 wells	Quantity for 96 wells	State	Vial cap color
Single-Break Strip Plate	48 wells	96 wells	Ready to use (precoated)	-
Dilution Microwells	48 wells	96 wells	Ready to use (red color)	-
Sealing film	2 sheets	2 sheets	Ready to use	-
Standards 1 - 6 (0, 0.05, 0.25, 0.5, 0.75 and 1.25 ppm of Almond in aqueous solution) (correspond to 0, 1, 5, 10, 15 and 25 ppm of Almond)	6 plastic vials (each 1.5 mL)	6 plastic vials (each 1.5 mL)	Ready to use	Brown
Standard Solution for Spiking 200 ppm of Almond	1 plastic vial (3 mL)	1 plastic vial (3 mL)	Ready to use	Brown
Almond detection solution	1 plastic vial (7,5 mL)	1 plastic vial (15 mL)	Ready to use	Green
Extraction buffer	2 plastic vials (60mL)	3 plastic vials (each 60mL)	5X Concentrate (dilute in distilled water)	Blue
Wash Buffer	1 plastic vial (50 mL)	1 plastic vial (50 mL)	20X Concentrate (dilute in distilled water)	White
TMB Substrate	1 plastic vial (7,5 mL)	1 plastic vial (15 mL)	Ready to use	Brown
Stop Solution	1 plastic vial (7,5 mL)	1 plastic vial (15 mL)	Ready to use	White

5. Materials required but not provided

- A grinder sufficient to render sample to particle size of fine instant coffee
- Balance with 0 - 50 g measuring capability and Graduated cylinder - 100mL
- Distilled or deionized water
- Centrifuge, centrifugal vials
- Vortex mixer and/or Shaker
- Microtiter plate reader fitted with 450 nm filter
- Water bath (60 °C / 140 °F)
- 100, 200 and 1000 µL adjustable single channel micropipettes with disposable tips (a repetitive pipette of 100µl is acceptable for the steps of TMB and Stop Solution)
- 50 - 300 µl multi-channel micropipette with disposable tips and reservoirs
- Skim Milk Powder

6. Storage Instructions

Store kit reagents between 2 and 8°C (35 - 46°F). Do not freeze any components provided. Reseal immediately the unused strips of the microtiter plate in the bag together with the desiccant bag provided and store at 2 - 8°C. After use remaining reagents should be returned to cold storage (2 - 8° C). Expiry of the kit and reagents is stated on the labels respectively and no quality guarantee is accepted after the expiration date. The expiry of the kit components can only be guaranteed if the components are stored properly as well as if the reagent is not contaminated by the first handling, in case of repeated use of one component. Because of the colorless TMB Substrate and standards light sensitivity, avoid the exposure to direct light. Do not interchange individual reagents between kits of different lot numbers.

7. Safety and Precautions for use

- Avoid any skin contact with standards (Almond proteins), Stop Solution (8% H_3PO_4) and TMB (toxic). **Use gloves.** In case of contact, wash thoroughly with water.
- All reagents should be warmed in room temperature before use and covered when not in use. **Use a clean disposable plastic pipette tip for each reagent, in order to avoid cross-contamination. When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.**
- Use a clean plastic container to prepare the wash buffer and all residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert the absorbent paper into the well. Read the absorbance within 60 minutes after completion of the assay.

8. Indication of corruption of kit reagents

- The bluish coloration of the chromogen substrate before the ELISA test.
- A value of less than 0.7 absorbance units (ABS 450nm) for the Standard 6 (St6).

9. Sample and Reagents Preparation

9.1 Reagents preparation

- Clean surfaces, glass vials, mincers and other equipment before and after each sample preparation.
- Dilute the 5X extraction solution concentrate 5 fold with distilled water to give a 1X working solution.

> **Preparation of Extraction Buffer 1X:** In case of the occurrence of crystals in the Extraction Buffer, the warming by gentle dismantling (using hands) of the crystals is needed. Only the amount which actually is needed should be diluted 1:5 (1+4) with distilled water (e. g. 50 ml concentrate + 200 ml distilled water, sufficient for the extraction of 20 samples). The clean bottle with **1X Extraction Buffer** working solution can be left out of the refrigerator during the method procedure and subsequently be stored 2 - 8°C for one month.

Note: The same extract can, also, be used in the following products: Allergen-Shield Brazil Nut A2948/A2998, Cashew A1948/A1996, Egg A1148/A1196, Hazelnut A1648/A1696, Lupin A2648/A2698, Macadamia A2748/A2798, Mustard A2848/A2898, Peanut A1748/A1796, Pecan A2048/A2096, Pine Nut A3048/A3098, Pistachio A2148/A2196, Sesame A2248/A2297, Walnut A2348/A2396.

- Dilute the 10X wash buffer solution concentrate 10 fold with distilled water to give a **1X** working solution.

> **Preparation of Wash Buffer 1X:** In case of the occurrence of crystals in the Wash Buffer, the warming by gentle dismantling (using hands) of the crystals is needed. Pour entire content of the solution concentrate (50ml) into a clean 500ml graduated cylinder, rinse the vial with distilled or deionized water and pour the content again into the cylinder and fill to a final volume of 500ml with distilled or deionized water. Mix gently to avoid foaming, transferring the final solution from cylinder to a clean bottle and back two times. The clean bottle with **1X Wash Buffer** working solution can be left out of the refrigerator during the method procedure and subsequent be stored 2 - 8°C for one month.

9.2 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 0.5g or 1gr ground portion of the sample and add 10mL or 20mL of the Extraction Buffer 1X respectively. Incubate for 10 min at 60°C by mixing them periodically on a vortex (e.g. per 2-3 min). **The ratio of sample to extraction solvent is 1:20 (w/v).**
- Allow the sample to cool down and centrifuge for 10 min, at least 2500 g, at room temperature (20 - 25 °C / 68 - 77 °F) and/or filter the extract (alternatively 2 ml of the extract can be centrifuged with high speed for 10 min in reaction caps by using a microcentrifuge).
- Transfer the supernatant in a screw top vial
- Use 100 µL directly in the immunoassay.

NOTE 1: The extracted sample should have a pH value of 6.2 - 7.5. If the pH is less than 6.2 or more than 7.5, the pH should be neutralized using NaOH or HCl.

NOTE 2: In case the user makes an additional dilution 1:1 of centrifuged sample with extraction buffer 1X the range of quantification becomes 0 - 50 ppm. Use 100 µL directly in the immunoassay. Multiply the final almond proteins ppm result x 2.

9.3 Liquid Samples

Use 0.5 mL of the sample, add 9.5 mL of the Extraction Buffer 1X and follow the rest of the procedure exactly as in step 9.2.

NOTE: For food matrices containing polyphenols, such as tannins, found in chocolate, fruits, wine, tea, coffee and cocoa, the addition of 5% skim milk powder to the extraction buffer is recommended to achieve optimal results.

10. Method procedure

10.1 Assay Design: Determine the number of microwell strips required to test the desired number of samples plus the appropriate number of wells needed for standards. Considering that each sample and standard can be tested in single or in duplicate, create a layout.

NOTE 1: If the number of wells is more than 32 (four strips), a repetitive pipette or multichannel pipette is necessary.

NOTE 2: It is recommended to test each sample and standard in duplicate considering Good Laboratory Practices and quality control requirements.

CAUTION: Use the standards positions in duplicate as the **Example plate** layout below **NECESSARY** and note positions of samples that can be set to all remaining empty wells of layout in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	St1	St1										
B	St2	St2										
C	St3	St3										
D	St4	St4										
E	St5	St5										
F	St6	St6										
G												
H												

Example plate layout (example for a 6 point standard curve)

10.2 Bring all reagents to room temperature (20 - 25°C) before use. Remove the **standards** (1 - 6) and place the **appropriate number** of Dilution Microwells (red) in a microwell holder for each Standard and Sample to be tested. Place an equal number of Antibody Coated Microtiter Wells in another microwell holder. Immediately reseal the unused strips of the microtiter plate in the bag together with the desiccant bag provided. The samples should be stored in a cool place.

10.3 Using a new pipette tip for each, transfer 150 µL of each standard (Standard 1 - 6) and prepared sample in duplicate (see 10.1) to the red-marked mixing wells.

10.4 Using a multichannel pipette, transfer 100µl of contents from each Dilution Microwell to a corresponding Antibody Coated Microtiter Well, cover the microwells with the sealing film, shake the microplate manually for 30 seconds and incubate at room temperature for **10 min**.

10.5 Remove the sealing film and wash the plate as follows: Aspirate the liquid from each well into the sink and tap the holder of microwells upside down strongly (four times in a row) on an absorbent paper to insure the complete removal of liquid from the wells. Dispense **300 µL** of **Wash Buffer 1X** (see 9.1) into each well with wash bottle or multichannel micropipette using the proper reagent reservoir and shaking the plate manually for a few seconds. Repeat this process for another three times (**total 4 times**).
CAUTION: It is important to not allow microwells to dry between working steps.

10.6 Aspirate the liquid as described above and add **100 µL** of **Almond Detection Solution** to each well. If the number of wells is more than 32 (four strips), a repetitive pipette or multichannel pipette is necessary (pour 1 mL of Detection Solution in a reservoir per 8 wells). Cover the microwells with the sealing film, shake the plate manually for a 30 seconds and incubate at room temperature for **10 min**.

10.7 Remove the sealing film and wash the plate as the wash step 10.5.

10.8 Aspirate the liquid as described above and add **100 µL** per well of **TMB Substrate** (pour 1mL per 8 wells in a reservoir). Cover the microwells with the sealing film, shaking the plate manually for a few seconds and incubate in the dark at room temperature for **10 min**.

10.9 Remove the sealing film and add **100 µL** per well of the **Stop Solution** to each well (pour 1 mL per 8 wells in a reservoir). Mix gently by shaking manually.

10.10 **Measure the absorbance at 450 nm.** Read the absorbance value of each well (within 60 minutes after the step 10.8) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

11. Data Analysis

- **Automatically**

An assigned software, the **Prognosis-Data-Reader,** is available for free (contact: exports@prognosis-biotech.com) download to evaluate the Allergen-Shield Almond ELISA kit. The evaluation is carried out by a simple transfer of data values after the measurement.

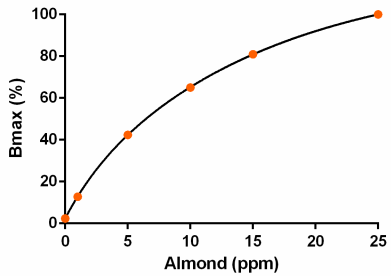
- **Manually**

Calculate the average absorbance values for each set of duplicate standards and samples. Ideally, duplicates should be within 10% of the mean. Use the following calculation:

$$\frac{\text{Standard or sample absorbance}}{\text{Standard 6 absorbance}} \times 100 = \%\ \text{Binding}$$

The almond content in each sample is determined by extrapolating OD values against the almond content of standard solutions using a fifth order polynomial standard curve.

12. Example of Standard Curve (0 - 25ppm)



13. Performance Evaluation

13.1 Reference Materials

Several reference materials are being used for the evaluation of each product of ProGnosis Biotech S.A. in the context of Quality Control performed by the Quality Control Department. Please request a validation report, including the results, at exports@prognosis-biotech.com.

13.2 Proficiency Tests

All products participate frequently in Proficiency Tests. For more information, visit the individual product page on our website: **www.prognosis-biotech.com**

14. Assay Claims

- Samples showing negative results may contain Almond below the limit of detection of the assay. This ELISA kit does not claim that food is safe for consumption based upon a determination of almond 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.
- Food samples that have been heat treated may contain denatured proteins which may not be captured by the antibody. Recovery of these matrices might be reduced.
- The protein content and the protein composition may differ among various species of the same matrix. Therefore, different varieties may produce different results.
- Test results are expressed as ppm almond. Almond contains around 21% protein. To express results as ppm almond protein, multiply the result by 0.21 (e.g., 1 ppm almond x 0.21 = 0.21 ppm almond protein).

15. Method Summary

Total procedure time (after samples and reagents preparation): 30 min.

Add 150µl of the samples and standards in the Dilution Microwells

⇩

Transfer 100 µL from each well of the Dilution Microwells into the Antibody Coated Microwells

⇩

Incubate 10 min at room temperature

⇩

Wash four times

⇩

Add 100 µl of ready-to-use Detection Solution and incubate 10 min at room temperature

⇩

Wash four times

⇩

Add 100 µl of ready-to-use TMB and let the color develop for 10 min in the dark at room temperature

⇩

Add 100 µl Stop Solution

⇩

Read Absorbance at 450 nm within 60 min

All immune assays supplied by ProGnosis Biotech S.A., are warranted to meet or exceed our published specification when used under normal conditions in your laboratory. If the product fails during the stated period, a replacement product will be issued.

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VERSION N8

CAT.NUMBER: A1548/A1596

STORAGE: 2-8°C

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