D0170 **ALMOND DIAGNOKIT**

The ingestion of certain proteins may result in serious allergic reactions in hypersensitive individuals whether children or adults. Reactions vary from simple urticaria to fatal anaphylaxis and only avoidance may be an effective means of protecting consumers.
Reaction to tree nuts such as almond is the second most prevalent food allergy affecting a substantial proportion of the population. Traces of almond proteins in manufactured foods may occur due to process cross-contamination.
Almond major protein (AMP or amandin), the primary storage protein in almonds, has been identified as the major almond allergen incriminated in IgE related reactions.

Almond DiagnoKit™ :
Quality control methodologies are required to identify whether almond allergens have made their way to the manufactured food products with no proper labeling. They are also useful to consumer protection agencies in order to enforce existing regulations on labeling of food ingredients.
Almond DiagnoKit™ is a sandwich immunoassay allowing the detection of almond proteins. An almond-specific antibody was raised targeting the major identified proteins. A suggested extraction and sample handling procedure was adapted to analyze chocolate samples. Almond DiagnoKit™ allows the immediate detection of almond proteins in chocolate samples.

Kit Description:
- Indirect enzyme-immunoassay for the detection of Almond protein.
- Suggested use: chocolate samples.

Test Principle: The test is based on a competitive binding of Almond labeled antibody, plate-immobilized and free almond proteins in a standard or sample solution.

Kit Content provided:
- Calculation diskette (MS Excel)
- 1 vial (1 mL) Almond Protein standard**100 µg/mL*
- 1 microtiter plate (12 strips of 8 wells) coated with Almond proteins.
- 1 vial (60µL) of almond antibody-HRP conjugate**.
- Washing buffer (dry powder)
- 10 mL Dilution buffer A (*to be used for the calibration curve only*)**
- Dilution buffer B (dry powder)
- Substrate Solution A, containing TMB (20 mL)
- Substrate Solution B, containing H2O2 ***(1 mL)
- Stopping reagent, containing H2SO4 ***(10 mL)

Contains 0.01% Thimerosal as preservative, consult MSDS.
****Corrosive, use with care*.**
**** Concentrations are based on a total protein amount determined by a BCA protein test***

Materials required, not provided:
- Precision adjustable pipet and a 12 or 8 channel multipipet able to deliver 200µL
- Plate reader with 450 nm interference filter
- Orbital shaker
- Vortex system
- Test tubes
- 500 mL squeeze bottle
- De-ionized water
- Timer

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Safety Precautions:
Items included in this kit are to be used by suitable qualified laboratory personnel, under proper laboratory working conditions. Handle all reagents and antibody in accordance with local safety procedures. Avoid any skin contact with stop solution and substrate B, in case of contact wash very well with water. Antibody-HRP solution contains thimerosal as preservative. Avoid contact of the reagent with the skin.

MSDS (Material Safety Data Sheets) available upon request.

Procedural notes:
Store the kit at 2-8 ºC. Before start the assay all reagents should be equilibrated at room temperature.
Return all reagents to 2-8ºC immediately after use.
Do not interchange reagents between kits of different lot numbers.
Do not use reagents beyond the expiration date of the kit.
Substrate solution is light sensitive. Avoid exposure to direct light, and avoid contact with metal, which can cause colour development.
A dark blue colour developed by the substrate solution after preparation is indicative of contamination. Sample extracts can be stored at 2-8ºC for seven days and at -20ºC for several months.

Preparation of Reagents
Prepare fresh diluted reagents, just prior to use
Washing Buffer*:*
Washing buffer is lyophilized and equivalent to 300 mL. Dissolve the dry powder in 30 mL of purified water for obtaining washing buffer **10 times more concentrated**; then dilute 1:10 with purified water the necessary volume.
Dilution Buffer A:
Ready to use.
Dilution Buffer B:
Dilution buffer B is lyophilized and equivalent to 100 mL. Dissolve the dry powder in 100 mL of purified water. Vortex for obtaining a clear solution if necessary.
Standard solutions*:*
Standard solutions should be prepared immediately prior to use in suitable glassware. Standard solutions may be obtained through the following dilution scheme: 40 µL of stock standard solution of 100 µg/mL + 960µL of **dilution buffer A** to obtain 4 µg/mL →**Standard 1**
Serial dilute 1 in 2 with **dilution buffer A**: 500µL of standard solution 1 + 500µL of **dilution buffer A**
(1mL total) to obtain:
Standard 2 → 2 µg/mL
Serial dilute 1 in 2 with **dilution buffer A**:
Standard 3 → 1 µg/mL
Standard 4 → 0.5 µg/mL
Standard 5 → 0.25 µg/mL
Standard 6 →0.125 µg/mL
Standard 7 → 0.0625 µg/mL
Almond Antibody-HRP conjugate*:*
Dilution **1:400** of the solution provided: Pipette 30 µL of almond antibody-HRP conjugate and dilute to 12 mL with dilution buffer B.
Substrate solution*:*
This solution should be prepared immediately prior to its use, by mixing the Solution A & B (10 mL of Substrate A with 5 µL of substrate B)
Stopping solution:
Ready to use.
Samples:
Samples should be diluted no less than 1:100 in Dilution buffer in order to avoid matrix effects.

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Test Procedure

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1. Prepare standards as described in **Preparation of Reagents.**

2. Using a precision pipet transfer 100 µL of each standard solution for calibration into a well on the plate, according with the following scheme (use 100µL of **Dilution Buffer A** in the Blank wells):

	1	2	3	4	5	6	7	8	9	10	11	12
A	***Blank 0µg/mL***	***Blank 0µg/mL***										
B	***Standard 0.0625µg/mL***	***Standard 0.0625µg/mL***										
C	***Standard 0.125µg/mL***	***Standard 0.125µg/mL***										
D	***Standard 0.250µg/mL***	***Standard 0.250µg/mL***			**Sample wells**							
E	***Standard 0.5µg/mL***	***Standard 0.5µg/mL***										
F	***Standard 1µg/mL***	***Standard 1µg/mL***										
G	***Standard 2µg/mL***	***Standard 2µg/mL***										
H	***Standard 4µg/mL***	***Standard 4µg/mL***										

3. Using a precision pipet transfer 100µL of each diluted unknown sample extract into assigned well (in duplicate or triplicate).

4. Shake the plate 5 minutes on orbital shaker

Addition of the Antibody enzyme conjugate

5. Using a precision pipet transfer 100µL of the diluted almond antibody-HRP conjugated solution, into each well.

Incubation on plate

6. Incubate the plate for 60 min. at room temperature in orbital shaker.

7. Empty the plate by inverting it over the sink then wash each well 5 times (Fill each well to the top with washing buffer, either with a squeeze bottle or a multichannel pipet. Turn the plate upside down and empty wells. The rinsing cycle should be carried out 5 times between incubation steps. Remove residual liquid by tapping the plate upside down on an absorbent paper).

8. Add 200µL of the substrate solution A+B to each well. Mix thoroughly and incubate for 20 minutes in the dark at room temperature.

9. Add 50µL of the stop solution to each well. Mix and incubate for 10 minutes in the dark at room temperature.

10. Take measurement of the absorbance with a plate reader at 450nm.

Results

An example of data processing is presented under a Micro Soft excel format and provided in the attached disk. A calculation table allows you to tabulate the mean O.D. for a duplicate run of standard solution.

Resulting graph will be suggested.

Data is treated so as the mean value of the absorbance (450nm) readings obtained for the standards and the samples are reported to the absorbance value of the zero standard.

$$\frac{\text{Absorbance standard (or sample)}}{\text{Absorbance zero standard}} \times 100 = \%\ B/Bo$$

Maximum OD Blank = zero standard

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A calibration curve can be obtained using the calculated % B/Bo value for each standard versus the log of the corresponding Protein concentration (in µg/ml).
Take the B/Bo (%) value for each sample and interpolate the corresponding concentration from the calibration curve. The linear transformation of this calibration curve may be obtained by plotting, logit (%B/Bo) versus ln C where:

$$\text{logit \% B/Bo} = \ln \frac{\%B/Bo}{100 - \%B/Bo}$$

In order to obtain the unknown concentration in µg/ml contained in a sample, use one of the linear ranges of the calibration curve of your choice. The determined value must be further multiplied by the corresponding dilution factor. This is based on the assumption that the recovery after extraction is 100%.
Positives may be considered certain when the O.D. obtained for a sample is 15% lower than that of the blank solution of the calibration curves. As matrix effect may appear, dilutions of sample may be beneficial. (A 1:100 dilution is suggested for the performance of this test on chocolate extracts). The standard diluent (Buffer A) was specially designed to mimic most commonly encountered matrices.

Suggested extraction for chocolate
Buffers:
PBS: Phosphate Buffered Saline (PBS adjusted to 7.4) contained 20 mmoL of NaH_2PO_4 and 140 mmoL of NaCl per liter of de ionized water.
Extraction Buffer:1.0 M NaCl, 0.1 M phosphate, 0.1% (w/v) Tween 20, 0.5% Triton x- 100, pH 7.0).

Material and Method:
1. Weigh out 10.0 g of sample into 250 mL screw top centrifuge tube.
2. Break up sample into smaller pieces.
3. Add 100mL extraction buffer to each sample (100 mL for 10.0 g sample).
4. Shake samples vigorously for one hour in a heated water bath set at 45 °C.
5. Centrifuge each sample at 3,000 rpm for 5 minutes.
6. Remove supernatant
7. Centrifuge supernatant for 30 minutes at 20,000 g in a refrigerated centrifuge at 4°C.
8. Filter the extract through Whatman # 1 filter paper and refrigerate.

Extracts will remain stable over a period of seven days at 4°C. For longer periods store at –20°C.

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