| VERSION 3

CAT.NUMBER: A1348/A1396

STORAGE: 2-8°C

Allergen Shield BLG

ELISA TEST | In vitro analysis

for the quantitative determination of beta-Lactoglobulin in food

PROGNOSIS BIOTECH

This is an electronic version, please verify always the last one included in the kit.

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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 BLG, A1348/A1396, is an immunoassay method that determines beta-Lactoglobulin in food products labeled as beta-Lactoglobulin-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:

Bakery products, beverages, bread mix, cake mix, non-dairy chocolate, cooked biscuit, cookies, corn flour, dressings, ice cream, infant breakfast cereal, infant soya formula, rice flour, sausage, soy products, wine

- Sample preparation: extraction
- Test time (incubation time after samples and reagents preparation): 30min
- Standard curve range: 0 - 4.5 ppm
- Shelf life: 12 months
- Storage: 2-8^{o}C

1. Description

Allergen-Shield BLG is an sandwich enzyme-linked immunosorbent assay (ELISA) test for the detection of beta-Lactoglobulin in food products labeled as beta-Lactoglobulin-free.

2. General Information

Cow`s milk contains 3.2 % proteins which consist of 10 % β-lactoglobulin (heat-resistant main fraction of whey proteins) and 80 % caseins. Consumption of β-lactoglobulin might be harmful for people who are allergic to milk. It is the most common protein that may cause an immune reaction to children. The allergen can be present as an ingredient or as a contamination in raw and cooked products due to the addition of whey or milk powder in many food products. Consumption of BLG-containing food from allergic people might cause a broad range of symptoms, such as hives, itching, mild oral allergy or/and anaphylactic shock. According to the regulation (EU) No. 1169/2011 Annex II, milk and therefore its ingredients are 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 beta-Lactoglobulin in a sample is determined by the immunological detection of beta-Lactoglobulin. The wells of the microtiter strips are coated with very specific antibodies against beta-Lactoglobulin. The standard solutions and the solutions of the samples are added and if a specimen contains beta-Lactoglobulin, the latter will bind to the immobilized antibodies. All of the unbound beta-Lactoglobulin proteins will be removed by washing. Then, the detection solution is added (peroxidase-conjugated antibody against beta-Lactoglobulin) and binds to beta-Lactoglobulin 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 beta-Lactoglobulin present in the samples and the standard solutions.

4. Reagents Provided

Allergen-Shield BLG 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 - 5 (0, 0.002 0.006, 0.019, 0.056 ppm of BLG in aqueous solution) (correspond to 0, 0.15, 0.5, 1.5 and 4.5 ppm of BLG)	5 plastic vials (each 1.5ml)	5 plastic vials (each 1.5ml)	Ready to use	Brown
BLG Detection Solution	1 plastic vial (8ml)	1 plastic vial (15ml)	Ready to use	Green
Extraction Buffer	1 plastic vial (60ml)	2 plastic vials (60ml)	5X Concentrate (dilute in distilled water)	Blue
Matrix Diluent	1 plastic vial (60ml)	2 plastic vials (60ml)	Ready to use	Yellow
Wash Buffer	1 plastic vial (50ml)	1 plastic vial (50ml)	20X Concentrate (dilute in distilled water)	White
TMB Substrate	1 plastic vial (8ml)	1 plastic vial (15ml)	Ready to use	Brown
Stop Solution	1 plastic vial (8ml)	1 plastic vial (15ml)	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 / 122 °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

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 (beta-Lactoglobulin), Stop Solution (15% 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 (450nm) for the Standard 5 (St5).

9. Reagents and sample 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. Make sure that the buffer is not contaminated with milk.

- Dilute the 20X wash buffer solution concentrate 20 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 the entire content of the solution concentrate (50ml) into a clean 1000ml 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 1000ml 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 subsequently 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 0.5 g ground portion of the sample, add 10 mL of the Extraction Buffer 1X and 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
- Dilute the sample 1: 3 (250 µL + 750 µL) with matrix diluent. The final dilution factor is 80.
- 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 - 9 ppm. Use 100 µL directly in the immunoassay. Multiply the final beta-Lactoglobulin 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.

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												
G												
H												

Example plate layout (example for a 5 point standard curve)

10.2 Bring all reagents to room temperature (19 - 24°C) before use. Remove the **standards** (1 - 5) 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 - 5) 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 **BLG 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 (immediately after the step 10.9 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: info@prognosis-biotech.com) download to evaluate the Allergen-Shield BLG ELISA kit. The evaluation is carried out by a simple transfer of data values after the measurement.

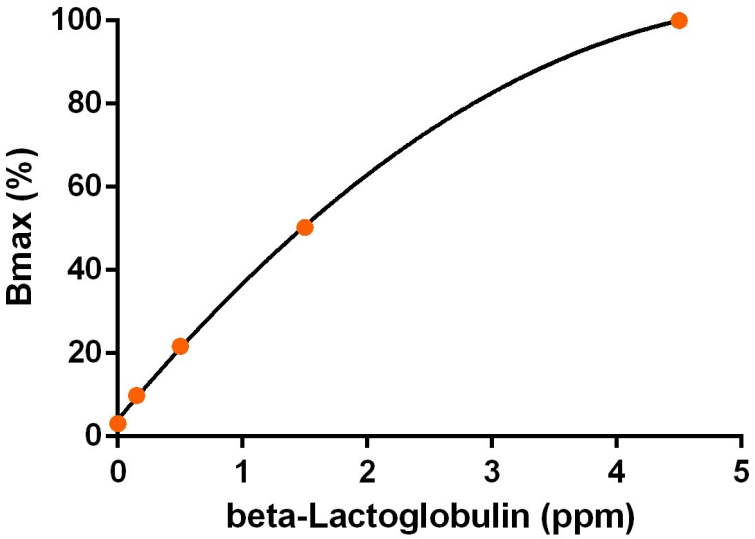
- **Manually**

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:

$$\frac{\text{Standard or sample absorbance}}{\text{Standard 5 absorbance}} \times 100 = \%\ \text{Binding}$$

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

12. Example of Standard Curve (0 - 4.5 ppm)



13. Immunoassay Specification

13.1 General Specification

- IC50 = 0.8 - 2.2 ppm.
- Each standards duplicates mean CV ≤ 6%.
- Coefficient of Variation (CV) of result at 1.5 ppm = 6.4% (n=16).

13.2 LOD - LOQ - Accuracy

- The LOD of the method is 0.1 ppm beta-Lactoglobulin.
- The LOQ of the method is 0.15 ppm beta-Lactoglobulin.
- The recovery of spiked extractions-matrices was 92.7% (CV = 7.1%).
- **Matrices:** Bakery products, beverages, bread mix, cake mix, non-dairy chocolate, cooked biscuit, cookies, corn flour, dressings, ice cream, infant breakfast cereal, infant soya formula, rice flour, sausage, wine

13.3 Specificity

- The antibodies specifically detect β-lactoglobulin of cow's milk. There is a cross reactivity to sheep's, goat's and buffalo's milk.
- The assay is specific for BLG and does not cross-react with nor recognizes other milk proteins such as casein.
- At concentrations as high as 500,000 ppm, no cross-reactivity was observed in other matrices (including tree nuts, peas, lentils, beans, legumes, seeds, flour (wheat, corn, oat, rice), egg, beef, fish and crustacea).

14. Performance Evaluation

14.1 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 info@prognosis-biotech.com.

14.2 Proficiency Tests

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

15. Assay Claims

- Samples showing negative results may contain BLG protein below the limit of detection of the assay, or they may contain casein and / or lactose. This ELISA kit does not claim that food is safe for consumption based upon a determination of milk 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.
- BLG represents about 10 % of the total milk protein, thus a 0.2 ppm BLG result corresponds to approximately 2 ppm milk protein.

16. Method Summary

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

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

Transfer 100µl from each well of the Dilution Microwells into the Antibody Coated Microwells and incubate 10min 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 and 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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