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Neogen Customer Assistance and Technical Service can be reached between 8 a.m. and 6 p.m. Eastern time by calling 800/234-5333 or 517/372-9200 and asking for a Neogen sales representative or Technical Services. Assistance is available on a 24-hour basis by calling 800/234-5333. Training on this product, and all Neogen test kits, is available.

MSDS INFORMATION AVAILABLE

Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's Food Safety test kits, at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

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T201185 BioKitsBLG-Assay_0510

Read instructions carefully before starting test

BioKits
BLG Assay Kit

Store at 2–8°C (35–46°F)

Enzyme immunoassay for the detection and quantification of β-Lactoglobulin (BLG) in food products and environmental swabs by enzyme immunoassay

SPECIFICATIONS

Format:	Indirect competitive enzyme immunoassay
Limit of Detection:	<2 ppm BLG The limit of detection (LOD) was statistically determined by extrapolation of the allergen concentration at an OD value of zero (the average OD value for the zero replicates) + 3 times the standard deviation of the OD results
Range of Quantification:	2.5–40 ppm
Units of Measurement:	BLG
Calibration:	NIST SRM8435 (whole milk powder)
No. of Determinations:	48 (including standards and controls)
Sample Preparation:	Buffer preparation, stomaching and centrifugation
Time Required:	Sample extraction time: approx. 40 minutes (5 samples) Test incubation time: 120 minutes
Specificity:	The polyclonal antibody specifically detects BLG
Cross-reactivity:	Not suitable for testing 100% egg products (raw or powder). This includes meringue based products where egg powder content is very high. In general where egg is an ingredient of a final product intended for consumption there is no cross-reactivity evident.

CALIBRATION

Units	Quantification
BLG	2.5–40 ppm
Total whey protein*	5–80 ppm

*Concentration will vary depending on source; see *Specificity* section.

SAFETY / COSHH NOTE

"Good laboratory practice" techniques should be employed when using this kit; if such practices are used the reagents constitute a very low potential risk to health. Safety clothing (lab coat, glasses and gloves if necessary) should be worn and skin contact with reagents avoided; do not ingest. Any contact with skin/eyes should be treated by washing/irrigation. It is also important to be aware of the allergic, toxic or infectious potential of analytical samples.

KIT COMPONENTS

Each kit contains sufficient material for 48 measurements (including standards and controls). The following components are provided in each kit:

Component	Detail	Vials / Bottle	Ready-to-Use
Milk Protein Standard		1	Requires dilution
BLG Control	USE WITH CARE: Contains high levels of BLG		✔
BLG Biotin		1	✔
Avidin Peroxidase Conjugate		1	✔
TMB Substrate		1	✔
Wash Solution Concentrate		1	10-fold concentrate
Diluent Concentrate Type 10		1	2-fold concentrate
Stop Solution	USE WITH CARE: STRONG ACID	1	✔
BLG Plate	48 microwells (6 x 8 strips + frame)	N/A	✔
Package Insert			
Worksheet			

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

- 0.05M carbonate / bicarbonate buffer pH 9.6 OR equivalent buffer tablets e.g. Sigma C3041
- Purified water
- Fish gelatin (Sigma G7765) optional

Equipment

- Ultra Turrax or similar homogenizer, and/or Waring or similar blender or food processor, and/or stomacher or similar homogenizer
- Miscellaneous laboratory plastic and/or glassware, including measuring cylinders, pipettes, disposable Pasteur pipettes, plate seals and containers suitable for food extracts
- pH meter
- Water bath capable of maintaining 60°C ± 2°
- Wrist action or similar shaker
- Validated microwell plate shaker set at 700–800 rpm
- Centrifuge and appropriate centrifuge/microfuge tubes for clarifying sample extract
- Precision micropipette(s) capable of delivering 50 and 100 microlitres, plus disposable tips
- Microwell washer (e.g. NUNC Immuno Wash 8) or wash bottle
- Microwell plate reader, fitted with 450 nm interference filter
  (Calibrate regularly. Fixed error <± 0.03; variable error <± 1%)

PREPARATION AND EXTRACTION OF SAMPLES

Note: The assay is extremely sensitive to the presence of BLG material. As an indication, <4 milligrams of BLG material can be detected in approximately one kilogram of food. However, such a limit of detection assumes that food samples can be ADEQUATELY SAMPLED prior to extraction and also that they can be rendered truly HOMOGENEOUS.

It is strongly recommended that due note is made of the order in which samples are processed so that the likelihood of a positive result that is actually a “false positive” (e.g. when a negative sample is processed immediately after a strongly contaminated one) can be assessed.

Because of the sensitivity of the method disposable tubes/containers should be used where possible and great care must be taken to thoroughly clean all reusable equipment, glassware, etc., between samples to avoid cross contamination.

PREPARATION OF SAMPLE EXTRACTION BUFFER

Note: The same sample extracts and sample extraction dilutions can be used for the following assay kits: Casein, BLG.

Prepare 0.05 M carbonate / bicarbonate buffer pH 9.6.

Prepare by mixing 0.05 M sodium carbonate/ 0.05 M sodium bicarbonate solutions to

pH 9.6 (± 0.1) OR Use Sigma tablets C3041. Make each tablet up to 100 mL (± 1 mL) with purified water.

Note: Improving extraction efficiency: Chocolate, cocoa, coffee and low protein samples can inhibit detection of milk proteins. Inhibition may be reduced if 10% fish gelatin (Sigma G7765) is added to the extraction solution prior to the sample extraction e.g.: 100 g buffer + 10 g of gelatin. Extraction is also improved if the mixture is heated to 50–60°C during extraction.

ENZYME IMMUNOASSAY PREPARATION

1. Prepare diluted sample extracts, controls and kit materials.
2. Remove all reagents from the kit box and allow to reach ROOM TEMPERATURE (18–22°C; 64–72°F) before starting the test.
3. **BLG biotin, Avidin peroxidase conjugate, TMB substrate, stop solution** are supplied ready to use, NO PREPARATION is necessary, simply mix by repeated inversion (do not shake).
4. **Milk protein standard preparation**

   Use the standard to prepare Working BLG Standards (2.5–40 ppm) in CLEAN glass/plastic tubes/vials. Standard (A) is prepared by removing the required MILK PROTEIN STANDARD volume indicated on the label, and diluting to a total volume 1000 µL. This first standard is then serially diluted to provide the standard curve as shown below:

Milk Protein Standard Solution		Working Diluent Solution	Final BLG Concentration (ppm)
A	Volume indicated on label made up to 1000 µL		40
B	500 µL (±5 µL) A	500 µL (±5 µL)	20
C	500 µL (±5 µL) B	500 µL (±5 µL)	10
D	500 µL (±5 µL) C	500 µL (±5 µL)	5
E	500 µL (±5 µL) D	500 µL (±5 µL)	2.5

The standard curve should be discarded after use and a fresh standard curve should be prepared prior to any further assays.

5. **Wash solution concentrate:** Supplied as a 10-fold concentrate. Dilute 1:9 in purified water to prepare working wash solution. For example add 100 mL (± 1 mL) to a volumetric flask/cylinder and make up to 1.0 liter (± 10 mL) with purified water.
6. **Diluent concentrate type 10:** Supplied as a 2 fold concentrate. Dilute 1:1 in purified water to prepare working diluent solution. For 48 microwells add 25 mL (± 0.5 mL) to a volumetric flask/cylinder, and make up to 50 mL (± 0.5 mL) with purified water. For any other number of microwells, dilute 1:1 with water, e.g. for a group of 24 microwells add 12.5 mL to 12.5 mL of purified water.

   Note: The diluent concentrate may produce crystals after refrigerated storage. **These crystals should be redissolved before use.** Warming to room temperature, with occasional mixing, should dissolve the salt crystals. If warming to room temperature is not

washing, contamination of the TMB substrate or splashing of Avidin peroxidase. Such imprecision is an indication of a problem during the performance of the assay, which may be INVALID and need to be repeated.

SPECIFICITY

The BLG Assay reacts predominantly with β-Lactoglobulin (BLG). Other types of BLG preparations may react somewhat differently in the assay:

Compound	% Cross-reactivity
β-Lactoglobulin	100
Whey Powder (Spike Control)	21.3
Whey Protein Concentrate	15.6
Caseins / Caseinates	0.7–1.3
α-Lactalbumin	0.4
Bovine Serum Albumin	0.1–0.3

Commercial whey preparations vary greatly in relative activities. This reflects the variety of such materials currently available: these range from crude whey powders (<10-20% by weight BLG) concentrates (~20–30% BLG) to very highly purified, high whey protein content products (>50% BLG).

The BLG Assay is an indirect, competitive assay. As such it detects not only intact BLG molecules but also, to varying degrees, damaged or degraded BLG fragments (e.g. from hydrolyzed milk preparations).

The nature of this mode of detection can throw up discrepancies between other detection methods, e.g. antibody capture “sandwich” assays, which detect larger fragments.

The antibodies used in the BLG Assay Kit also react to a small extent with BLG from sheep and goats.

Because of this fact, and the extremely low limit of detection of the BLG assay, the determination of bovine BLG/whey content or detection of the degree of adulteration in non-bovine dairy products is technically complex.

Milk from cows, sheep, goat and buffalo can also be detected using F.A.S.T. Immunostick and PCR-based products.

For more information on dairy protein quantitation, dairy product adulteration testing or these alternative methods, please contact Neogen or your local distributor.

ESTIMATION OF BLG PROTEIN CONTENT FROM SWAB SAMPLES

Approximate estimates of the quantity of BLG present in the swab solution are taken from the BLG calibration curve as follows:

A swab sample (no dilution) giving an absorbance which extrapolates to 10 ppm on the BLG standard curve contains 100 ng/mL BLG. This factor of x10 and conversion to ng/mL can be applied to any undiluted swab sample which gives an absorbance value which falls within the quantifiable range (2.5–40 ppm).

Because of the variability of the swabbing process, the amount of detectable protein in the swabbing solution may vary considerably. Recoveries of various allergens from a swabbed area vary quite widely and detection of allergens/BLG from complex and/or highly processed food sources can be difficult. For further information see the BioKits Allergen Swabbing Kit Insert.

SHELF LIFE

Diluted wash buffer: Once diluted 1:9 the wash buffer is stable at room temperature in a sealed clean container for at least 1 week.

Diluted assay diluent: Fresh assay diluent should be prepared for each assay.

Extraction buffer: Fresh extraction buffer should be prepared daily.

Extracted samples: The undiluted sample extracts may be stored at 2–8ºC (35–46°F) for up to five days. If prolonged storage is required the undiluted extracts must be kept frozen (< 20ºC) where they are stable for several months.

Kit reagents: The kit should be stored at 2–8ºC (35–46°F). The shelf life of unopened kit components is indicated by the expiry date on the respective labels. Once the kit reagents have been opened, exposure to elevated (i.e. room) temperatures should be minimized.

Antibody sensitized plate must be kept dry. Keep sealed in foil pouch with desiccant.

Providing these instructions are complied with the opened kit reagents should be stable for many weeks or months at 2–8ºC (35–46°F).

PERFORMANCE CHARACTERISTICS

- The assay is designed to give optimum performance at ambient temperature (19–23ºC; 66–73°F)
- Maximum binding (Bzero; zero standard) absorbance should be greater than 1.5 absorbance units (A450 nm)
- Standard A (40 ppm): the %B/Bzero for the 40 ppm standard should be below 20%
- Standard E (2.5 ppm): there should be a slight but visible difference in color between the zero and the 2.5 ppm BLG standard (%B/Bzero 2.5 ppm = 70–95%)
- BLG control should read off the standard curve within the specifications printed on the certificate of analysis that accompanies this kit
- At temperatures below 19ºC or above 23ºC incubations may need to be lengthened or reduced respectively to maintain performance

Poor replication (e.g. six zero standard wells %CV >12.5–15%) may indicate inadequate

sufficient, then warming to 40°C with mixing will be required.

7. **BLG Plate:** Open the foil pouch. Take out the microwell module, remove the microwell strips not required and return them to the pouch taking care that the desiccant is present; **reseal the pouch carefully.**

   Note: With a pencil, number the columns in sequence on the upper frosted edge of the strips in use; this preserves the identity of the strips should they become detached from the frame.

PREPARATION AND EXTRACTION OF SAMPLES / CONTROLS

Sample the material to be tested; prepare by stomaching, grinding, blending or mincing. It is important to reduce the particle size of the material as far as possible, if possible to a flour or paste, while avoiding the potential for cross-contamination.

1. Weigh out 2 g (±0.02 g) of solid or 2 mL (±20 µL) of liquid sample into a suitable container.

Controls

Assay Control (recommended)

Dilute 25 µL of BLG control in 5 mL of extraction buffer. This dilution must be diluted a further 1/10 in working diluent, prior to assay. This produces a kit control that should read off the standard curve at approximately 10 ppm BLG.

Spike Control

Users may wish to spike 2 g of a sample with 100 µL of BLG control. Mix well and set aside for at least 30 minutes while preparing other reagents. Extract and dilute spiked sample as described below. This will prepare a spiked assay control sample containing the equivalent of ~10 ppm BLG content.

2. Add 20 mL (±0.2 mL) extraction solution.
3. Homogenize, stomach or blend sample for 120 seconds (± 10 seconds).
4. Remove a portion of the extracted sample slurry with a disposable Pasteur pipette, place into an Eppendorf tube, seal and centrifuge at 500xg for 10 minutes (± 1 minute) at room temperature, or alternatively allow the mixture to settle, to obtain a liquid supernatant layer (the sample extract).
5. With a clean disposable Pasteur pipette, remove a portion of the aqueous extract from below any fat that might be present and above the settled layer; place into a second, clean tube/container. Mix/vortex well.
6. Dilute the settled/centrifuged sample extracts 1:10 in Working Diluent Solution. Add 50 µL (± 0.5 µL) of extract to 450 µL (± 5 µL) of Working Diluent in a clean glass/plastic container. Mix/vortex well.
7. The diluted sample extract is now ready for testing.

   Note: Samples which are known to contain, or are suspected of containing whey proteins, or which give results above the top standard at a 1/10 dilution (βLG >400 ng/mL = >40 ppm), need to be diluted further to obtain values within the range of the standard curve.

ENVIRONMENTAL SWAB SAMPLE PREPARATION

For the preparation of environmental swab samples the BioKits Allergen Swabbing Kit (BASK) is required (Cat No. 901042J). This kit can be used in conjunction with the BLG Assay Kit for the determination of BLG contamination levels in the environment.

TEST PROCEDURE

It is recommended that, for practice assays, small runs are performed and duplicate wells used for all samples. When good precision is being achieved (replicate OD450 nm %CVs < ~15%) reaction wells may be run singly. However, it is good laboratory practice that duplicates are run for some or all diluted extracts and imprecision (%CV of OD450 nm and concentration values) estimated in all assays as part of an ongoing QC program.

Note: When testing has been started, all steps should be completed without interruption to reduce the possibility of assay "drift" across the microwells.

1. Prepare diluted sample extracts and kit materials.
2. Add 100 µL (± 1 µL) of working diluent (used as zero standard), BLG standards (A–E), controls and each of the diluted sample extracts into appropriate microwells.

   Note: Use a separate disposable tip for pipetting each standard/diluted sample extract to prevent cross-contamination.
3. IMMEDIATELY ADD 50 µL (± 0.5 µL) of BLG biotin to all wells.
4. Place the microwell plate on a plate shaker and incubate with vigorous shaking at room temperature for 60 MINUTES (± 1 minute).
5. Discard the liquid from the microwells, then (using NUNC Immuno Wash 8 or wash bottle) completely fill all wells with working wash solution. Discard the liquid and repeat the fill and discard sequence 4 more times (5 washes in total). Following the final discard, tap the plate upside down on several layers of absorbent tissue to completely remove residual droplets/bubbles of wash solution.
6. Add 50 µL (±0.5 µL) of Avidin peroxidase conjugate to all wells. Work from top to bottom of each strip as previously described.
7. Place the microwell plate on a plate shaker and incubate with vigorous shaking at room temperature for 15 MINUTES (± 1 minute).
8. At the end of the incubation, repeat the wash sequence described in Step 5.
9. Add 100 µL (± 1 µL) of TMB substrate to all wells.
10. Mix the strips gently by hand on a flat surface and incubate for 45 MINUTES at room temperature, static (without shaking).

    Note: The rate of color development is dependent on laboratory conditions and should be monitored in order to obtain suitable OD450 nm levels.
11. Add 50 µL (± 0.5 µL) of stop solution to all wells. Mix gently by hand to distribute the stop solution and prevent further color development. Color changes from blue to yellow and intensifies.

12. Using a microplate reader fitted with a 450 nm filter blank the reader on "Air" then measure and record the absorbance of each of the microwells.

    Note: Readings should be completed within 10 minutes of adding stop solution.

RESULTS

Qualitative

For qualitative assessment an individual BLG standard can be used to define a specific (X ppm) cutoff level from the average OD450 nm.

Samples with absorbance values below the cutoff are classified as: POSITIVE >X ppm

Samples at or above the cutoff are classified as: NEGATIVE <X ppm

Quantitative

Quantitative estimates of BLG content can be obtained by using a calibration curve; to construct the calibration curve, use two-cycle log graph paper. Plot the mean absorbance value for each of the BLG standards (2.5, 5, 10, 20 and 40 ppm) and fit a best curve to join each neighboring point. Alternatively, the results can be calculated using a graphical data reduction package using a suitable line fit for the curve.

Values returned when interpolating off the BLG assay curve line correspond approximately to BLG content in the ORIGINAL SAMPLE (assuming that the nominal extraction and dilution conditions are adhered to). If additional dilutions are performed then the necessary factor needs to be applied to the recorded BLG content.

Note: Indirect competitive enzyme immunoassay - Absorbance will be seen to decrease with increasing concentration of BLG. See example standard curve on enclosed Certificate of Analysis.

Report samples with an OD450 > standard E as "<LOQ". Report samples with an OD450 < standard A as ">40 ppm".

Poor replication (e.g. six zero standard wells %CV >12.5–15%) may indicate inadequate washing, contamination of the TMB substrate or splashing of Avidin peroxidase. Such imprecision is an indication of a problem during the performance of the assay, which may be INVALID and need to be repeated.

INTERPRETATION

The variability of raw material/product sampling, food composition (salinity, acidity, etc.) treatment of foodstuffs during processing (heat, pressure, etc.), difficulty of obtaining complete homogeneity during extraction and the reactivity of different sources of BLG material means that the amount of detectable BLG protein in the extract may vary considerably.

Note: If a food sample gives a negative result in the test **it may still contain BLG material** which is either unreactive in the test or below the limit of detection. **It should not be assumed that the food is "BLG free".**