Read instructions carefully before starting test

BioKits
Walnut Assay Kit

Store at 2–8°C (35–46°F)

Sandwich enzyme immunoassay for the detection and quantification of Walnut in food products and environmental swabs by enzyme immunoassay

SPECIFICATIONS

Limit of Detection:	0.25 ppm walnut The theoretical LOD was statistically determined by extrapolation of the allergen concentration at an OD value of zero (the average OD value for the blank replicates, over 10 separate assays) + 3 times the standard deviation of the OD results
Range of Quantification:	2.4 – 120 ppm
Units of Measurement:	Walnut
Calibration:	Kjeldahl analyzed (N x 6.25) total protein extraction (Juglans regia); No certified reference material available
No. of Determinations:	48 (including standards and controls)
Sample Preparation:	Buffer preparation, shaking and centrifugation
Time Required:	Sample extraction time: approx 40 minutes (5 samples) Test incubation time: 75 minutes
Specificity:	The polyclonal antibody specifically detects walnut proteins
Cross-reactivity:	Of a large panel of commodities including nuts, pulses, grains, proteins and seeds the following were reactive: pecan (2.3%); quinoa (0.0012%); pistachio (0.0009%); hazelnut (0.0005%); buckwheat (0.00024%)

CALIBRATION

Units	Quantification
Walnut	2.4 to 120 ppm
Walnut Protein	0.4 to 20 ppm
Conversion Factor (% Protein)	6 (~17%*)

* Sze-Tao K. W. C. and Sathe S. K. (2000)

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
Walnut Standard S1	2.4 ppm Walnut	1	✔
Walnut Standard S2	6 ppm Walnut	1	✔
Walnut Standard S3	12 ppm Walnut	1	✔
Walnut Standard S4	60 ppm Walnut	1	✔
Walnut Standard S5	120 ppm Walnut	1	✔
Walnut Biotin		1	✔
Avidin Peroxidase Conjugate		1	✔
TMB Substrate		1	✔
Wash Solution Concentrate		1	10-fold concentrate
Diluent Concentrate Type 8		1	5-fold concentrate
Stop Solution	**Caution:** STRONG ACID	1	✔
Walnut plate	48 Microwells (6 x 8 strips + frame)	N/A	✔
Package insert			
Blank result form			

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents (all Analytical or equivalent grade)

1. Sodium chloride
2. Tris (hydroxymethyl) methylamine
3. 1M hydrochloric acid
4. Teleost gelatin (Sigma G7765)

Equipment

1. Pestle and mortar, stomacher or suitable grinder/mill, which can be easily cleaned after use to eliminate the possibility of cross-contamination between samples
2. Miscellaneous laboratory plastic and/or glassware, including measuring cylinders, pipettes, disposable Pasteur pipettes, plate seals and containers suitable for food extracts
3. pH meter
4. Water bath capable of maintaining 60°C ± 2°C
5. Wrist action or similar shaker
6. Centrifuge and appropriate centrifuge/microfuge tubes for clarifying sample extract
7. Precision micropipette(s) capable of delivering 50 and 100 microliters, plus disposable tips

8. 96 well plate seals
9. Microwell washer (e.g. NUNC Immuno Wash 8) or wash bottle
10. 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 walnut material. As an indication, <2.4 milligrams of walnut 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 homogenous.**

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" (i.e. 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 and 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: Egg and Peanut.

Prepare high salt Tris extraction buffer for the extraction of food samples. Amounts quoted are for 1.5 liters, the volume required for 28 samples (~55 mL per sample to be extracted).

1. Dissolve 9.1 g (± 0.1 g) Tris and 17.5 g (± 0.1 g) sodium chloride in ~1L purified water.
2. Add 150 g gelatin (Sigma G7765) and stir well to dissolve.
3. Adjust pH to 8.15–8.25 with 1M hydrochloric acid; make up volume to 1,500 mL (± 10 mL).

ENZYME IMMUNOASSAY PREPARATION

1. Prepare diluted sample extracts, controls (see pg. 3) 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. **Walnut standards, walnut biotin, Avidin peroxidase conjugate, TMB substrate and stop solution** are supplied ready to use. No preparation is necessary; simply mix by repeated inversion (do not shake).
4. **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.
5. **Diluent concentrate type 8**: Supplied as a 5-fold concentrate. Dilute 1:4 in purified water to prepare working diluent solution. For 48 microwells add 30 mL (± 0.5 mL) to a volumetric flask/cylinder, and make up to 150 mL (± 1.5 mL) with purified water. For any other number of micro-wells, dilute 1:4 with water, e.g. for a group of 24 microwells add 15 mL (± 0.2 mL) to 60 mL (± 1 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 sufficient, then warming to 40°C with mixing will be required.
6. **Walnut 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 material by stomaching, grinding, blending or mincing. It is important to reduce the particle size of the material as much as possible, if possible to a flour or paste, while avoiding the potential for cross-contamination.

1. Pre-heat extraction buffer to 60°C in a water bath.
2. Weigh out at least 5 g of each sample into a clean container. If appropriate (e.g. for chocolate) melt sample in water bath before adding hot extraction buffer.
3. Add hot extraction buffer at a ratio of 10 mL (± 0.1 mL) per gram of sample into the container containing sample to be extracted.
4. Shake for 15 minutes at room temperature.
5. Remove a portion of the extracted sample slurry with a disposable Pasteur pipette, then place into an Eppendorf tube or other tube. Seal and centrifuge at ~10,000 g for 10 minutes. Alternatively, allow the mixture to settle until a liquid extract layer appears. **Note**: during validation, recoveries of walnut protein were found to be higher when centrifugation was employed.
6. 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.
7. Dilute the settled/centrifuged sample extracts 1:1 in working diluent solution, e.g. add 500 µL (± 5 µL) of extract to 500 µL (± 5 µL) of working diluent in a clean glass/plastic container. Mix/vortex well.
8. The diluted sample extract is now ready for testing.

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 Walnut Assay Kit for the determination of walnut 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 %CV's < ~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), walnut standard(s) S1–S5, 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. Incubate for 30 minutes at room temperature without shaking.
4. 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.
5. Add 50 µL (± 0.5 µL) of walnut biotin to all wells.
6. Mix the strips in the frame gently by hand on a flat surface and allow to incubate for 15 minutes at room temperature, static.
7. At the end of the incubation, repeat the wash sequence used in Step 4.
8. Using a micropipette or preferably a repeating pipette with clean tip, add 50 µL (± 0.5 µL) of Avidin peroxidase conjugate to appropriate (non-blank) wells. Work from top to bottom of each

strip as previously described. Leave blank wells empty.

9. Mix the strips in the frame gently by hand on a flat surface and allow to incubate for 15 minutes at room temperature, static.
10. At the end of the incubation, repeat the wash sequence described in Step 4.
11. Add 100 µL (± 1 µL) of TMB substrate to all wells.
12. Mix the strips gently by hand on a flat surface and incubate for 15 minutes at room temperature 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.
13. 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.
14. 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 walnut 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: Negative < X ppm

Samples at or above the cutoff are classified as: Positive > X ppm

Quantitative

Quantitative estimates of walnut 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 walnut standards (2.4, 6, 12, 60 and 120 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 walnut assay curve line correspond approximately to walnut 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 walnut content.

Report samples with an OD450 < standard S1 as "<LOQ." Report samples with an OD450 > standard S5 as ">120 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 walnut material means that the amount of detectable walnut protein in the extract may vary considerably.

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

ESTIMATION OF WALNUT PROTEIN CONTENT FROM SWAB SAMPLES

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

A swab sample (no dilution) giving an absorbance which extrapolates to 10 ppm on the walnut standard curve contains 500 ng/mL walnut (83 ng/mL walnut protein). The following conversion can be applied to any undiluted swab sample that gives an absorbance value that falls within the quantifiable range (2.4–120 ppm):

reported walnut concentration (ppm) ÷ 0.02 = walnut residue in swab solution (ng/mL)
walnut residue in swab solution (ng/mL) ÷ 6 = walnut protein in swab solution (ng/mL)

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/walnut 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 5 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

1. The assay is designed to give optimum performance at ambient temperature (18–22ºC; 64–72°F).
2. Standard S1 OD450 nm should be greater than 2 x zero OD450nm
3. Standard S5 OD450 nm preferably >1.5 absorbance units.
4. At temperatures below 18ºC or above 22ºC, 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 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.

REFERENCES

Sze-Tao K. W. C. and Sathe S. K. (2000). Walnuts (Juglans regia L) : proximate composition, protein solubility, protein amino acid composition and protein in vitro digestibility. J Sci Food Agric 80:1393-1401.

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Services can be reached by calling 1-800-234-5333. Training on this product, and all Neogen test kits, is available.

SDS INFORMATION AVAILABLE
Safety data sheets (SDS) are available for this test kit, and all of Neogen's test kits, on Neogen's website at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

TERMS AND CONDITIONS
For Neogen's full terms and conditions, please visit www.neogen.com/Corporate/termsconditions.html.

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TESTING KITS AVAILABLE FROM NEOGEN

Natural toxins
- Aflatoxin, DON, ochratoxin, zearalenone, T-2/HT-2 toxins, fumonisin, histamine

Foodborne bacteria
- *E. coli* O157:H7, *Salmonella*, *Listeria*, *Listeria monocytogenes, Campylobacter, Staphylococcus aureus*, *Salmonella enteritidis*

Sanitation
- ATP, yeast and mold, total plate count, generic *E. coli* and total coliforms, protein residues

Food allergens
- Almonds, crustacea, eggs, gliadin, hazelnut, milk, mustard, peanut, sesame, soy, walnut, multi-treenut

Genetic modification
- CP4 (Roundup Ready®)

Ruminant by-products
- Meat and bone meal, feed

Species identification
- Raw and cooked meat samples, feed

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