HAZELNUT-CHECK

ELISA KIT

For the quantitative determination of low levels of

HAZELNUT

in raw materials, environmental swabs, processed foods etc.

INSTRUCTIONS FOR USE

READ CAREFULLY BEFORE PROCEEDING!

Cat. No. **R6015 (48-wells) / R6014 (96-wells)**

STORE REFRIGERATED (2-8°C – see Section 5.2.1)

QIS188 HAZELNUT ELISA R6014 R6015 V02 SEPTEMBER 2011

Amendments: Units of measurement now **PPM (mg/kg) HAZELNUT**

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INSTRUCTIONS FOR USE: CONTENTS

Section	Page	TITLE
1.	3	INTRODUCTION TO THIS TEST KIT
2.	3	INTENDED USES OF THE KIT
3.	4	DIAGRAM OF THE ELISA METHOD
4.	4	SAMPLE PREPARATION & ELISA OVERVIEW
5.	5	SAFETY & PROCEDURAL NOTES
6.	6	KIT MATERIALS PROVIDED
7.	7	EQUIPMENT & MATERIALS YOU NEED (NOT INCLUDED)
8.	8	PREPARATION OF EXTRACTION SOLUTION & REAGENTS
9.	8	PREPARATION OF SAMPLES/CONTROLS/STANDARDS
10.	9	EXAMPLE ASSAY LAYOUT
11.	10	ELISA PROCEDURE
12.	11	CALCULATION OF RESULTS
13.	11	EXAMPLE ASSAY DATA
14.	12	INTERPRETATION OF RESULTS
15.	13	PERFORMANCE INDICATIONS
16.	13	PROBLEM SOLVING
17.	15	RECYCLING

1. **INTRODUCTION TO THIS TEST KIT**

1.1. The prevalence of food allergy is increasing, age at onset is decreasing and the condition can have severe effects, including breathing difficulties, anaphylaxis and in rare instances, death. Accordingly, legislation has been put in place to ensure that allergy sufferers are informed of the *intentional inclusion* of key food allergens. In the EC, Directives 2003/89 & 2005/26 became fully implemented in November 2005 and other allergens were added in 2006. In the USA, the “Food Allergen Labeling and Consumer Protection Act” (FALCPA) came into force early in 2006; both mandate the clear labelling of particular food allergens.

1.2. In the **EC** the intentional presence of the following foods must be listed in the ingredients: celery/celeriac; cereals (*wheat*, rye, barley, oats, spelt, kamut or their hybridised strains); *crustacea, eggs; fish*; lupin, molluscs (gastropods, bivalves, cephalopods), mustard; sesame; *soya* & *tree nuts* (almond, hazelnut, walnut, cashew, pecan, Brazil, pistachio, macadamia/Queensland). In the **USA** the list is similar but includes only those foods in *italics* above.

1.3. Food producers are being advised by food protection agencies such as the UK’s FSA and the US FDA to restrict the use of so-called “may contain” labelling to products whose manufacturing processes have been assessed using HACCP and for which the presence of undeclared allergens is both significant and unavoidable.

1.4. Hazelnut allergies represent a significant proportion of tree nut related acute allergic reactions; the major allergenic proteins in hazelnut are *Cor a9* and *Cor a11*. Hazelnut products are increasingly prevalent in a range of processed foods particularly confectionery, snacks and baked goods thereby increasing the risk of exposure to allergy sufferers.

2. **INTENDED USES OF THE KIT**

2.1. The assay utilises polyclonal antibodies to detect the mixture of proteins present in soluble extracts of hazelnut using a direct “sandwich” ELISA technique.

2.2. It is designed to detect hazelnut proteins at very low levels in fresh, processed and heated food products as well as in environmental swabs.

2.3. The assay range is nominally between 1-40 mg/kg (PPM) hazelnut, which corresponds to ~0.2-7ppm hazelnut protein if the stated extraction ratio is used and samples are not diluted. The range can be extended upwards by amending these ratios if required.

2.4. The Limit Of Detection (LOD) of the assay is ~0.3PPM hazelnut, which corresponds to <0.1 PPM hazelnut protein.

3. DIAGRAM OF THE ELISA METHOD

1. 2. 3. 4.

1. Antibody coated plastic micro-well.

2. Hazelnut (▲) in food extract binds to antibody on well.

3. HRP enzyme-labelled antibody binds in turn to hazelnut bound to antibody on well.

4. Addition of TMB substrate, which develops blue colour in presence of HRP enzyme.

4. SAMPLE PREPARATION & ELISA OVERVIEW

4.1. Laboratory Sample Preparation

⇩ **Prepare** Sample by grinding/chopping/blending until homogeneous.

⇩ **Add** 1 part Test Portion to 20 parts Extraction Buffer 1X.

⇩ **Extract** Incubate for 15 minutes @ 60°C Shaking every two minutes.

⇩ **Separate** Centrifuge for 10 minutes.

4.2. ELISA Protocol

⇩ **Pipette** **100µL** Standards, Sample Extracts into wells.

⇩ **Mix. Incubate** at room temperature for **20 minutes**.

⇩ **Wash wells** three times with Wash Solution.

⇩ **Pipette** **100µL** anti-hazelnut peroxidase Conjugate into wells.

⇩ **Mix. Incubate** at room temperature for **20 minutes**.

⇩ **Wash wells** three times with Wash Solution.

⇩ **Pipette** **100µL** TMB Substrate reagent into wells.

⇩ **Mix. Incubate** at room temperature in the dark for **20 minutes**.

⇩ **Pipette** **100µL** Acid Stop Solution into wells.

⇩ **Mix. Read** wells at **450nm** wavelength within 15 minutes.

⇩ **Calculate** PPM Hazelnut results for all Samples.

5. SAFETY/PROCEDURAL NOTES

5.1. SAFETY

5.1.1. Some of the kit contents contain a low concentration of the preservative thiomersal (thimerosal; merthiolate), however the kit is safe if used according to these instructions and Good Laboratory Practice (GLP) techniques.

5.1.2. Stop Solution contains a relatively weak concentration of sulphuric acid: wear safety glasses; use with care; avoid splashing.

5.2. PROCEDURAL

5.2.1. **Store kit at 2-8°C.** Wash Solution concentrate may form crystals/precipitates, *which must be re-dissolved e.g.by placing the bottle in warm water,* prior to use.

5.2.2. Users should maintain normal standards of good laboratory practice.

5.2.3. If not stated, tolerances required for the various measurements used are:

Temperature ± 1°C; Time ± 1 minute; Volumes & Weights ± 1%

5.2.4. Because of the extreme sensitivity of the test, very high standards of cleanliness should be observed when handling Laboratory Samples/Test Portions, using equipment and cleaning down before, between and after all stages in the process. The use of a swabbing kit (e.g. Imutest Swabbing kits Cat. No. A6008/A6009) can help validate laboratory/ equipment cleaning regimes.

5.2.5. Proteins bind strongly to some plastics e.g. polystyrene; it is recommended that polypropylene or glass are used for sample handling.

5.2.6. To prevent cross-contamination, pipette tips should not be reused.

5.2.7. “Reverse” pipetting is preferred for air displacement pipettes; rinse tip several times before pipetting out. Avoid drops of reagent on the outside of the tip entering wells by wiping carefully with a clean tissue.

5.2.8. The key to good results is consistency from sample to sample & well to well; work quickly but carefully to avoid assay drift; small (32/48 well) assays are preferred. Duplicate wells (at least some!) are strongly recommended.

5.2.9. If required for re-testing, Laboratory Sample extracts can be stored FROZEN at or below -18°C; they remain stable for several weeks.

6. **KIT MATERIALS PROVIDED** (Note: Kit contents vary slightly depending on kit size i.e. 48- or 96-well presentations – see below).

6.1. **Extraction & Sample Dilution Buffer 10X Concentrate**

6.1.1. 2 x 30mL or 4 x 30mL provided; diluted (1X) Extraction & Sample Dilution buffer is used to extract and, if necessary, dilute the Laboratory Samples/Extracts.

6.2. **Anti-hazelnut antibody-Coated microwell Plates** (6 or 12 x 8 well strips).

6.2.1. Foil sealed in a re-sealable pouch with a harmless, self-indicating (yellow: active; green: not active – do not use plate) desiccant.

6.3. Pre-prepared **Standards**.

6.3.1. 5 x 2.0mL or 5 x 3.0mL each of five ready to use Standards containing Zero, 1, 5, 15 & 40 PPM Hazelnut.

6.3.2. NOTE: If additional "cut-off" Standards are required for e.g. Qualitative testing, use Extraction/Dilution Buffer as the Zero Standard and also use it to dilute one of the higher Standards to a suitable cut-off value.

6.4. **Wash Buffer 10X Concentrate**

6.4.1. 1 x 30mL or 2 x 30mL to be used at 1X to wash the assay wells.

6.5. **Anti-Hazelnut peroxidase Conjugate**

6.5.1. 1 x 7.5mL or 1 x 15mL of Conjugate.

6.6. **TMB Substrate**

6.6.1. 1 x 7.5mL or 1 x 15mL; ready to use. CARE: Light Sensitive (turns blue!)

6.7. **Stop Solution** CAUTION: Dilute acid

6.7.1. 1 x 7.5mL or 1 x 15mL of dilute H_2SO_4

6.8. **Instructions For Use & Assay Layout Guide**

7. **EQUIPMENT & MATERIALS**

7.1. WHAT YOU MAY NEED (NOT INCLUDED IN THE KIT):

7.2. Sample mill, chopper or blender for Laboratory Sample preparation – requirement dependent on sample type – see Sample Preparation below.

7.3. Two place balance.

7.4. Centrifuge capable of achieving at least 1000g; 2000g – 5000g is preferable. The use of a centrifuge capable of directly spinning extraction tubes saves time and reduces the possibility of cross-contamination between extracts.

7.5. Purified water for reagent preparation.

7.6. Clean containers for making up and containing 1X Extraction buffer & Wash solution.

7.7. Polypropylene or glass containers for test portion extraction (~20-30mL), and centrifugation (if possible use extraction tube).

7.8. Microlitre pipettes and tips.

7.9. Wash bottle with fine spout and absorbent paper towel for microwell washing.

7.10. ELISA plate reader with 450nm wavelength filter.

7.11. **OPTIONAL MATERIALS/EQUIPMENT:**

7.12. The use of a repeating pipette capable of delivering multiples of 100μL volumes, plus dispensing syringes and tips, helps speed the addition of HRP Conjugate, TMB Substrate & Stop reagents, thus minimising assay drift.

7.13. The use of an automated or hand-held ELISA plate washer system reduces the time taken to wash plates and can improve consistency.

7.14. ELISA software greatly reduces the time required to calculate results.

8. **PREPARATION OF KIT REAGENTS**

8.1. Allow kit contents to reach room temperature before preparing reagents; if a precipitate/crystals form in Wash Solution concentrate, warm slightly and mix well to re-dissolve before dilution.

8.2. Extraction & Sample Dilution Buffer: Dilute 1/10 (1:9) with purified water (e.g. add 15mL concentrate to 135mL water and mix well).

FOR SOLID SAMPLES: Add e.g. 0.5 g of HOMOGENEOUS sample to 10 ml of the prepared Extraction & Sample Dilution Buffer.

FOR LIQUID SAMPLES: Add e.g. 0.5 ml of HOMOGENEOUS sample to 9.5 ml of the prepared Extraction & Sample Dilution Buffer.

8.3. Prepare Wash Solution 1X by diluting Wash Solution 10X Concentrate 1/10 (1:9) with purified water (e.g. add 15mL concentrate to 135mL water and mix well).

8.4. Prepare anti-hazelnut antibody Coated Plates by cutting carefully across the foil pouch above the resealing “zip” from one notch to the other.

8.4.1. Take out the number of strips required, place them in the white strip holding frame; place the cover on the strips until they are required for use.

8.4.2. Replace the remaining strips in the foil pouch with the desiccant; seal carefully ensuring that the zip is properly secured.

9. **PREPARATION OF LABORATORY SAMPLES**

9.1. Swabbing samples are assayed undiluted.

9.2. Finely divided flours/powders, fine breadcrumbs and smooth liquids require no preparation.

9.3. For non-homogeneous Samples, e.g. sausages and other meat products, take out several grams of a representative portion of the sample and prepare by milling, grinding, chopping, blending etc until it has a fine particle size and/or appears to be homogeneous.

9.4. Weigh out a Test Portion of 0.5g into 10.0ml of Extraction Buffer 1X in e.g. a polypropylene tube/universal container. Record the exact weight added – you do not have to add exactly 0.5g, but make sure you record the weight and correct back for the actual weight used when calculating results.

9.5. Place the sample into a pre heated water bath at 60°C for 15 minutes shaking every two minutes to maintain homogeneity.

9.6. Either pour a portion of the extract into a suitable centrifuge tube or, if possible, spin the whole tube at ≥2,000g for 10 minutes. Alternatively, allow to settle for at least 30 minutes or until a reasonably clear layer appears above the settled food.

9.7. If a fatty layer appears above the extraction solution it is best to remove it by careful aspiration with a vacuum line.

9.8. If further dilution is required for any extracted samples dilute with Extraction Buffer 1X, which can also be used as the Zero Standard if required.

IMPORTANT NOTE: Test Portion extracts prepared for the following Allergen-Check ELISAs:

Almond; β-LG; Egg; Hazelnut; Lupin; Mustard; Peanut; Sesame; Soya; Walnut

can be used in **ANY** of these assays, reducing operator time substantially when one sample is being tested for multiple allergens.

10. EXAMPLE ASSAY LAYOUT

10.1. Suggested Assay Layouts (32 & 48 well assays).

	4 Strip/32 Well assay						6 Strip/48 Well Assay					
	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	U2	S0	U8			U1	U1	S0	U9	U9	S0
B	S1	U3	S1	U8			U2	U2	S1	U10	U10	S1
C	S2	U3	S2	U9			U3	U3	S2	U11	U11	S2
D	S3	U4	S3	U9			U4	U4	S3	U12	U12	S3
E	S4	U4	S4	U10			U5	U5	S4	U13	U13	S4
F	U1	U5	U6	U10			U6	U6	U17	U14	U14	U17
G	U1	U5	U7	U11			U7	U7	U18	U15	U15	U18
H	U2	U6	U7	U11			U8	U8	U19	U16	U16	U19

Key to Layout:

S0 – S4

Hazelnut Standards (Zero-40ppm)

U1 – U19

Sample Extracts

11. DETAILED ELISA PROCEDURE

11.1. Allow all kit reagents to reach room temperature (18-24°C preferable); prepare Test Portion extracts and all ELISA reagents as described above.

11.2. Ensure that the work area is well organised and tidy, all extracts are clearly labelled in the correct order (Layout Guide) for pipetting and that ELISA equipment is ready for use; remove caps from all Standards/extracts to speed up addition to the wells.

11.3. Mark microwell strips on upper or lower tab to keep them in the correct order should they become detached from frame.

11.4. Mix the ready to use STANDARDS, Conjugate, TMB and STOP reagents gently just before use.

11.5. Add 100µL of Standards and Sample Extracts to the appropriate wells using a microlitre pipette.

11.6. Mix the plate by sliding back and forth, gently but briskly, in short movements (1-2cm side to side) on a smooth surface.

11.7. Cover the plate and incubate at room temperature for **20 minutes**.

11.8. WASHING: Empty wells by flicking out contents into a sink; carefully fill each well in turn using a wash bottle containing 1x Wash Solution. Repeat emptying and filling cycle twice more. After the THREE wash cycles, flick out the plate several times to remove excess water; tap the wells upside down FIRMLY on absorbent paper until little or no liquid appears on the paper; while inverted, wipe base of wells to clean them.

Alternatively: Use a hand held/automatic plate washer to aspirate then fill wells THREE times with 1x Wash Solution; tap onto paper and clean base as described above.

11.9. Immediately add 100µl of Anti-Hazelnut peroxidase Conjugate reagent using a microlitre or repeating pipette; mix as described in 11.6.

11.10. Cover the plate and incubate at room temperature for **20 minutes**.

11.11. Wash all wells THREE times with 1x Wash Solution as in 11.8.

11.12. Immediately add 100µl of TMB Substrate to all wells; mix as described in 11.6.

11.13. Cover plate; incubate at room temperature for **20 minutes IN THE DARK** (e.g. in a drawer), or until sufficient colour develops (sections 15/16; p. 13/14).

11.14. Add 100µl of Stop Solution to all wells (blue to yellow colour change in wells).

11.15. Mix plate as described in 11.6 to stop enzyme activity and evenly distribute colour. (Colour remains stable for up to 15 minutes)

11.16. Read plate at 450nm using the plate reader and record absorbance values.

12. CALCULATION OF RESULTS

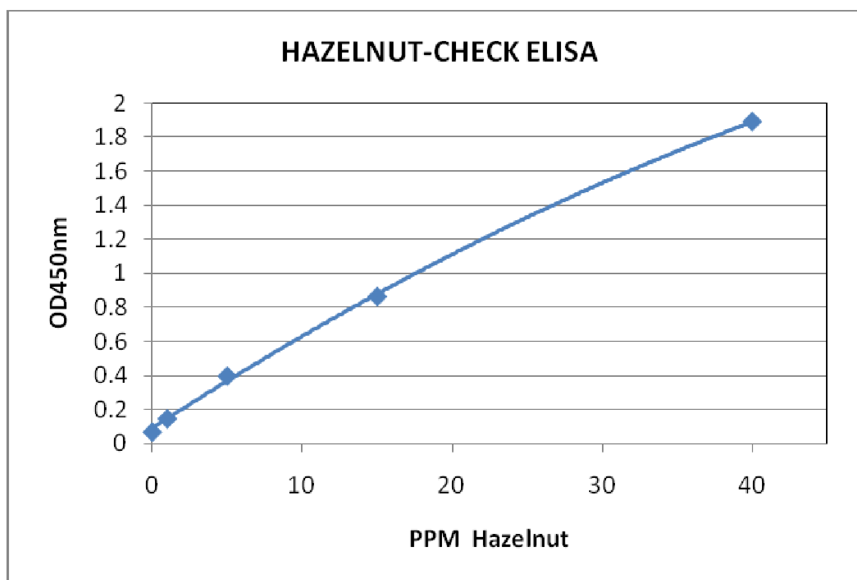
12.1. Plot Standard Curve on normal or semi-log graph paper; draw a line/curve of best fit; read off unknown Sample concentrations (PPM); record results on Layout Guide. Alternatively use curve-fit software to produce the results.

12.1.1. Plot OD450nm values (Y-axis) against PPM Hazelnut values (X-axis; 0.0, 1.0, 5.0, 15.0, 40.0, PPM)

12.2. IMPORTANT NOTE: Remember to correct for the actual sample weights and dilutions used during the calculation process if exact weights/volumes were not used.

13. EXAMPLE ASSAY DATA

13.1.	Zero Standard:	OD_{450}	0.070
13.2.	1.0 ppm	OD_{450}	0.148
13.3.	5.0 ppm	OD_{450}	0.398
13.4.	15.0 ppm	OD_{450}	0.865
13.5.	40.0 ppm	OD_{450}	1.891



14. INTERPRETATION OF RESULTS

14.1. Assay Calibration. At present there is no agreed calibrator to help support Hazelnut allergen analysis. The Imutest Hazelnut-Check ELISA has been calibrated using a panel of Hazelnut free/low Hazelnut spiked (incurred) food samples.

14.2. Recovery after spiking of Hazelnut Standard into four matrices was as follows:

14.2.1.	Cookies	99%
14.2.2.	Cereal	99%
14.2.3.	Ice cream	89%
14.2.4.	Dark chocolate	81%

14.3. Cross reactivity: the antibody used in this kit did not react with:

Sunflower seeds	Pea
Poppy seeds	Chickpea
Rye	Bean
Corn	Pumpkin seeds
Barley	Walnut
Oats	Pistachio
Rice	Brazil nut
Wheat	Pine nut
Soya	Chestnut
Soya lecithin	Macadamia
Sesame	Cashew nut
Buckwheat	Peanut
Cocoa	Coconut
Dried cow’s milk	Beef gelatine
Apple	Gluten
Almond	

15. PERFORMANCE INDICATIONS

15.1. Prior to stopping the ELISA, S0 wells should be nearly colourless and there should be a definite colour difference between the S0 and pale blue S2 (5.0ppm) wells. The S4 (40 ppm) wells should be a mid blue colour.

15.2. Indicative assay parameters are suggested to be as follows:

15.3. Zero OD_{450}nm: <0.20 units

15.4. Limit of Detection: <0.5 PPM (at 3 x Std. Dev. from Zero)

15.5. 5 ppm OD_{450nm}: >4.0 x Zero OD450nm

15.6. 40 ppm OD_{450nm}: >1.0 units; preferably >1.5 units.

15.7. Duplicate precision (OD_{450nm}): Ideally <5%

15.8. Duplicate precision (PPM Hazelnut): Ideally <10 – 15%

16. PROBLEM SOLVING

16.1. Regular maintenance and calibration of equipment helps improve assay performance.

16.2. Good laboratory practice reduces the possibility of cross contamination; swabbing kits (e.g. Imutest's Swabbing kits Cat. No. A6008 & A6009) can help validate and verify laboratory/equipment cleaning regimes.

16.3. Poor replication is most often due to poorly maintained pipettes or inadequate/inconsistent plate washing.

16.4. Pipettes: ensure that all pipettes are kept in good condition and are regularly calibrated.

16.5. Try to avoid bubbles in the wells during the last wash by carefully overfilling, especially when using a wash bottle. If using a hand-held washer the bubbles can be aspirated away; if using a wash bottle, flick out well contents vigorously. After washing, tap **vigorously** on absorbent paper towel until no bubbles remain in the wells and little or no liquid appears on the paper towel.

16.6. Consistently attaining ideal levels of colour development (see example data section 13; page 11) depends on:

16.6.1. laboratory temperature; at temperatures below 18°C incubation times tend to be longer and above 22°C they may need to be shortened.

16.6.2. effectiveness of washing; ensure that wells are filled to the rim and remember, it is difficult to over-wash!

16.6.3. plate reader range; some readers can measure up to an absorbance as high as 3 units or more, whereas others are limited to <2 units. It is important to judge colour development to fit the range of your reader.

16.6.4. previous experience of this ELISA in your lab.

16.6.5. age of the kit (proximity to expiry date).

16.7. If you also have a 620nm filter you can monitor colour development after ~nine minutes to help predict final (stopped) OD450nm values. These will be ~3 times the predicted OD620nm level at ten minutes. Stop the assay when the OD620 value is expected to be between 0.5–0.8 units.

16.8. If your plate reader has a pre-mixing facility, set the speed to between 700-900 cycles per minute and time for ~20 seconds.

17. RECYCLING

Wherever possible, D.I.L. recycles its waste materials. Please help our environment by recycling the paper/card, plastic & glass used in this kit and during the extraction & dilution processes.

Remember the recycling mantra:

- ✓ Reduce
- ✓ Reuse (with care – avoid cross contamination!)
- ✓ Recycle

Diagnostic Innovations Limited ensures that its products are made from high quality raw materials but can make no warranty, express or implied, as to their suitability other than to measure allergen content when used exactly in accordance with these instructions.

Use of the kit for any other purpose is outside its intended use.

Any damages, including consequential or special damage or expense arising directly or indirectly from using this product are limited to the replacement value of the kit.

Other Imutest allergen/gluten detection related products are also available:

A range of **Allergen-Check & Gluten-Check ELISA kits** for:
peanut, almond, hazelnut, walnut, milk (caseins), milk (BLG/whey), mustard, cereal/wheat gluten, egg, lupin, soya, crustacea, (white) fish & sesame

Allergen/Gluten Environmental & Surface **Swabbing Kits**

GLUTEN FlowThrough™ & GLUTEN SWABBING Tests
For gluten swabbing/testing in small laboratories or in food manufacturing plants without laboratory facilities for e.g.
Raw material & finished product testing (detects 10-20PPM gluten)
Environmental swabs (detects <1ppm gluten in the swab solution)

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