

imutest

FISH-CHECK

ELISA KIT

For the quantitative determination of low levels of
FISH (esp. Gadiformes) PROTEINS
in raw materials, environmental swabs, processed foods etc.

INSTRUCTIONS FOR USE

***** AMENDED IFU *****

***** READ CAREFULLY BEFORE PROCEEDING! *****

Cat. No. **R6009 (48)/R6010 (96)/R6011 (192)**

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

QIS200_IFU FISH E R6009_10_11_V02

August 2011

**Amendments: 1. USE OF ANTI-FISH HRP reagent instead of Biotin/Avidin.
2. CHANGE to INCUBATION TIMES (now 40+40+15 minutes)**

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1. INTRODUCTION TO THIS TEST KIT

- 1.1. The prevalence of food allergy is increasing, the 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 in many countries to ensure that allergy sufferers are informed of the intentional inclusion of key food allergens. In the EC, Directives 2003/89 & 2005/26 have been fully implemented and more allergens added. 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; *peanuts*; 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. Fish products are widespread in the food supply and fish proteins can be potent, even life-threatening, allergens. Those with fish allergies are usually sensitive to several different fish species, although levels of the key allergen (parvalbumin) have been reported to vary widely across species¹.

2. INTENDED USES OF THE KIT

- 2.1. The assay utilises ELISA techniques and detects the presence of cod parvalbumin (the major fish allergen). Imutest's FISH-CHECK ELISA kits are designed for the detection of a variety of fish species at low levels.
- 2.2. For fresh COD, the assay has a nominal range of 5-125 mg/kg (PPM), in e.g. raw materials and finished food products. The reactivity of other species and of processed (e.g. cooked, canned, pickled) foods may be somewhat different.
- 2.3. Assay originally developed² by the Norwegian Veterinary Institute (NVI); the kits are manufactured by D.I.L. in the U.K. under license from the NVI.

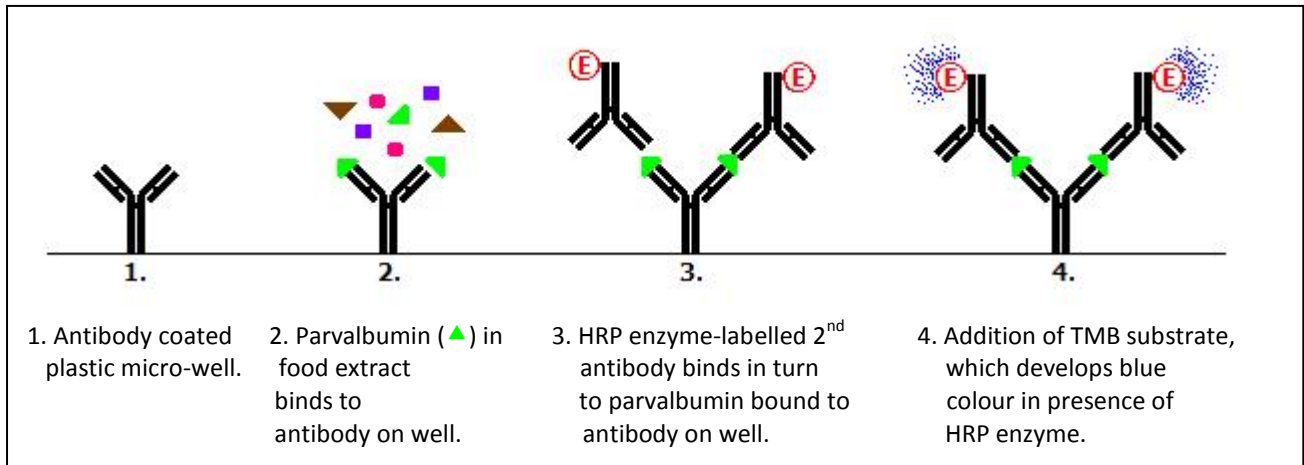
¹ Kuehn et al, Important variations in parvalbumin content in common fish species; Int. Arch. All. Immunol. 153 (2010) pp359-366.

² Faeste & Plassen, Quantitative sandwich ELISA for the determination of fish in foods; J. Immunol. Meth. 329 (2008) pp45-55.

(Copies available on request)



3. DIAGRAM OF THE ELISA METHOD:



4. SAMPLE PREPARATION & ELISA OVERVIEW

4.1. Laboratory Sample/Kit Controls Preparation

- ↓ **Prepare** Sample by grinding/chopping/blending until homogeneous.
- ↓ **Add** Test Portion of **0.5g** to 4.5ml of Extraction Solution.
- ↓ **Mix. Extract** 15 minutes to extract fish.
- ↓ **Mix. Centrifuge** 10 minutes or allow to **settle** for 30 minutes.
- ↓ **Remove** a portion of the extract above the food pellet.
- ↓ **Dilute** extracts 1/5 (1:4) in Diluent Solution 2 (1x).

4.2. ELISA Protocol

- ↓ **Pipette** 100µL Standards and diluted Sample/Control Extracts into wells.
- ↓ **Mix. Incubate** at room temperature for **40 minutes**.
- ↓ **Wash wells** three times with wash solution 1X.
- ↓ **Pipette** 100µL Anti-Fish HRP into wells.
- ↓ **Mix. Incubate** at room temperature for **40 minutes**.
- ↓ **Wash wells** four times with wash solution 1X.
- ↓ **Pipette** 100µL TMB Substrate reagent into wells.
- ↓ **Mix. Incubate** at room temperature for **15 minutes**.
- ↓ **Pipette** 100µL Acid Stop Solution 1 into wells.
- ↓ **Mix. Read** wells at **450nm** wavelength.
- ↓ **Calculate** mg/kg (PPM) Fish results for all Controls/Samples.



5. SAFETY/PROCEDURAL NOTES

5.1. SAFETY

- 5.1.1. There are NO toxic ingredients/preservatives in any of the kit contents, therefore the kit is safe if used according to these instructions.
- 5.1.2. Acid Stop Solution 1 contains a relatively weak concentration of sulphuric acid (0.5M/1N): wear safety glasses; use with care; avoid splashing.

5.2. PROCEDURAL

- 5.2.1. **Store kit reagents at 2-8°C.**
- 5.2.2. Storage of Diluent/Wash Concentrates at room temperature reduces the formation of crystals (which must be re-dissolved prior to use).
- 5.2.3. Users should maintain normal standards of good laboratory practice.
- 5.2.4. If not stated, tolerances required for the various measurements used are:
Temperature $\pm 1^{\circ}\text{C}$; Time ± 1 minute; Volumes & Weights $\pm 1\%$
- 5.2.5. 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. Homogeniser tools are difficult to clean properly; use of semi-disposable tools can speed the extraction process but the shake/heat method is preferable to reduce cross-contamination. Use of a swabbing kit (e.g. imutest ESS A6008 [100 sets]/A6009 [25 sets]) can help validate lab./equipment cleaning regimes.
- 5.2.6. Proteins bind strongly to some plastics e.g. polystyrene; it is recommended that extraction & dilution tubes are polypropylene or glass. 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/Control extracts can be stored refrigerated (2-8°C) for 2-3 days or frozen ($<-18^{\circ}\text{C}$) for up to 3 months.
- 5.2.10. If you require extra Wash or Diluent concentrate e.g. if using an automated washer and/or performing a number of high dilutions of extracts, please contact D.I.L. or your local distributor for additional free supplies.



6. **KIT MATERIALS PROVIDED** (Note: Kit contents vary slightly depending on kit size i.e. 48-, 96- or 192-well presentations – see below).
- 6.1. **Kit Control Concentrate (5X).**
- 6.1.1. 1.4mL of Cod Fish Control, with yellow dye as a pipetting aid; **to be diluted 1:4 (1/5)** with Diluent Concentrate Type 2 (1X) prior to assaying.
- 6.1.2. For (PPM) Cod content, see Certificate Of Analysis sheet in each kit.
- 6.2. **Diluent Concentrate 2 (5X).**
- 6.2.1. 1 x or 2 x 25mL; to be diluted with purified water.
- 6.3. Pre-prepared **Cod Parvalbumin Standards. READY FOR USE**
- 6.3.1. 1.4mL each of 5 Parvalbumin Standards, with red dye as a pipetting aid.
- 6.3.1.1. **5 mg/kg (PPM):** **BLUE** colour coded cap.
- 6.3.1.2. **10 mg/kg (PPM):** **GREEN** colour coded cap.
- 6.3.1.3. **20 mg/kg (PPM):** **YELLOW** colour coded cap.
- 6.3.1.4. **50 mg/kg (PPM):** **ORANGE** colour coded cap.
- 6.3.1.5. **125 mg/kg (PPM):** **RED** colour coded cap.
- 6.4. **Anti-Fish (Parvalbumin) Microwell Plate** (1 x 48; 1 x 96 or 2 x 96 wells).
- 6.4.1. Foil sealed in a re-sealable pouch with a harmless desiccant (yellow or yellow/green if still active; green if exhausted – **do not use plate**).
- 6.5. **Wash Solution Concentrate (20X).**
- 6.5.1. 1, 2 or 4 x 25mL; to be diluted with purified water.
- 6.6. **Anti-Fish (Parvalbumin) HRP reagent.**
- 6.6.1. 1 x or 2 x 12mL or 1 x 24mL; ready to use; contain food dye as pipetting aid.
- 6.7. **TMB Substrate**
- 6.7.1. 1 x or 2 x 12mL or 1 x 24mL; ready to use. CARE: LIGHT SENSITIVE (turns **blue!**)
- 6.8. **Acid Stop Solution 1**
- 6.8.1. 1 x 24mL of 0.5M H₂SO₄; ready to use.
- 6.9. Laboratory **Sample Preparation & Assay Layout Guide** form.
- 6.9.1. Photocopy as required; write in assay layout & use as a pipetting guide.



7. EQUIPMENT & MATERIALS

7.1. WHAT YOU NEED (NOT INCLUDED):

- 7.2. Sample mill, chopper or blender for Laboratory Sample preparation.
- 7.3. 2-Place balance to weigh out Test Portions.
- 7.4. Purified water for Extraction, Diluent & Wash solution preparation.
- 7.5. Glycine and Tris-hydroxymethyl-methylamine to prepare Extraction Solution.
- 7.6. As a time-saving **ALTERNATIVE: use DIL's imutest Allergen Extraction Kit 1** (Cat. No. A6015; 24 ready to use tubes each containing the above allergen extraction solution with yellow dye as a pipetting aid).
- 7.7. Containers for making up and containing diluent/wash solutions and extractions.
- 7.8. Polypropylene or glass containers for test portion extraction (~20-30mL; not required if using A6015), centrifugation (if possible use extraction tube) and dilution (~2-5mL).
- 7.9. Microlitre pipettes and tips (100µl and 400µl).
- 7.10. Wash bottle with fine spout and absorbent paper towel for microwell washing.
- 7.11. ELISA plate reader with 450nm wavelength filter.

7.12. OPTIONAL MATERIALS/EQUIPMENT:

- 7.13. Homogeniser for Test Portion extraction.
- 7.14. Centrifuge capable of achieving at least 1000g; 2000g is preferable. The use of a centrifuge capable of directly spinning extraction tubes (e.g. A6015 tubes) saves time and reduces the possibility of cross-contamination between extracts.
- 7.15. The use of a (100µL) repeating pipette, dispensing syringes and tips helps speed addition of HRP, TMB & Stop reagents, minimising assay drift.
- 7.16. The use of an automated or hand-held ELISA plate washer system reduces the time taken to wash plates and can improve consistency.
- 7.17. ELISA software greatly reduces the time required to calculate results.



8. PREPARATION OF THE EXTRACTION SOLUTION & KIT REAGENTS

8.1. If not using **A6015**: Prepare **Extraction Solution** (5mL needed for each Sample + some excess). For EIGHTEEN Samples:

8.1.1. Add 3.75g Glycine; 1.21g Tris to ~90ml purified water and mix well; when dissolved adjust to pH 8.65-8.75 then make up to 100mL (102g). The solution is stable for 2-3 weeks if stored refrigerated; MIX WELL prior to use.

8.1.2. Note: Specific Gravity ~1.02g/mL.

8.1.3. Add 4.5mL (4.6g) of solution to one tube for each Laboratory Sample.

8.2. Prepare Diluent Solution 2 (1X) & Wash Solution (1X)

8.2.1. Note: Diluent Concentrate 2 and Wash Solution Concentrate can form crystals if stored refrigerated. Help avoid this by removing from kit and storing at Room Temperature, or warm prior to use to re-dissolve any crystals which may have formed.

8.2.2. Dilute Diluent Concentrate 2 (5X) at a ratio of 1:4 (1/5) in purified water, or add bottle contents to 100mL of water.

8.2.3. Dilute Wash Solution Concentrate (20X) at a ratio of 1:19 (1/20) in purified water, or add bottle contents to 475mL of water

8.3. Anti-Fish Microwell Plate (48/96 wells)

8.3.1. Cut or carefully tear foil pouch between the two notches; pull open pouch closure and remove plate. Carefully remove strips not required for the ELISA from the plate frame.

8.3.2. Re-seal remaining wells in pouch by carefully pressing the re-sealable closure across its width; ensure that desiccant is present and active (yellow or yellow to pale green in colour).

8.4. Anti-Fish HRP Reagent

8.4.1. Minimise the time the HRP reagent is at room temperature.

8.4.2. Keep out of strong light conditions.



9. PREPARATION OF LABORATORY SAMPLES/CONTROLS/STANDARD CURVE

9.1. SWABBING & LABORATORY SAMPLES/KIT CONTROLS

- 9.1.1. imutest-ESS swab samples can be assayed undiluted – proceed to 11.1.
- 9.1.2. Finely divided flours/powders, fine breadcrumbs and smooth liquids require no preparation – proceed to 9.1.4.
- 9.1.3. For non-homogeneous Samples, take out 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.
- 9.1.4. Weigh out Test Portion of ~0.50g into 4.5ml (4.6g) of Extraction Solution in e.g. a polypropylene tube/universal container – **you do not have to add exactly 0.50g** but **DO** record the exact weight added on Layout Guide.
- 9.1.5. Shake vigorously by hand or on a mixer for TWO minutes to disperse Test Portion in the extraction solution.
- 9.1.6. Allow to stand at Room Temperature for 15 MINUTES.
- 9.1.7. Shake vigorously again for TWO minutes.
- 9.1.8. Either pour a small portion of the extract into a suitable centrifuge tube or, if possible, spin the whole tube. Centrifuge at $\geq 1,000g$ for 10-15 minutes. Alternatively, allow to settle for at least 30 minutes or until a reasonably clear layer appears above the settled food.
- 9.1.9. If a fatty layer appears above the extraction solution it is best to remove it by careful aspiration with a Pasteur-type pipette using a bulb or vacuum line.
- 9.1.10. Carefully pipette a 100 μ L portion of extract from the liquid layer above the food pellet and add to 400 μ L (0.400mL) of 1X Diluent Solution 2. Mix well.
- 9.1.11. Carefully pipette 100 μ L of the Kit Control and add to 400 μ L (0.400mL) of 1X Diluent Solution 2. Mix well.

NOTE: When using a 1:10 extraction ratio and 1/5 dilution the (parvalbumin) standard curve equates to ~5-125 mg/kg (PPM) fresh cod – see also Section 12. page 12.

Swabbing solutions can be further diluted in Diluent 2 (1X) if required.

For foods expected to contain higher levels of fish, dilute the initial 1/5 dilution of test portion extract by a further:

- ⇒ 10-fold (standard curve equates to 50 to 1,250 PPM).
- ⇒ 100-fold (standard curve equates to 500 to 12,500 PPM) etc.

- 9.1.12. The diluted extract is now ready for ELISA testing (section 11; page 11).



9.2. STANDARD CURVE

- 9.2.1. Use 1X Diluent 2 as the Zero standard.
- 9.2.2. The Standards provided (5, 10, 20, 50 & 125mg/kg cod equivalent) are ready for use in the ELISA procedure. Mix gently before use.
- 9.2.3. The Standards have a gradation of red colour from 5-125 Standards as a visual aid during pipetting.

NOTE: If using A6015 the yellow dye present allows instant recognition of those extracts that have been diluted. For most foods, A6015 Extract dilutions should be a pale yellow colour.

10. EXAMPLE ASSAY LAYOUT

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

4 Strip/32 Well assay						6 Strip/48 Well Assay						
A	S0	U2	S0	U6	○	○	U1	U1	S0	C	C	S0
B	S1	U2	S1	U6	○	○	U2	U2	S1	U10	U10	S1
C	S2	U3	S2	U7	○	○	U3	U3	S2	U11	U11	S2
D	S3	U3	S3	U7	○	○	U4	U4	S3	U12	U12	S3
E	S4	U4	S4	U8	○	○	U5	U5	S4	U13	U13	S4
F	S5	U4	S5	U8	○	○	U6	U6	S5	U14	U14	S5
G	U1	C	U5	U9	○	○	U7	U7	U9	U15	U15	U17
H	U1	C	U5	U9	○	○	U8	U8	U9	U16	U16	U17
	1	2	3	4	5	6	7	8	9	10	11	12

Key to Layout:

S0 – S5

Parvalbumin

Standards

(Zero-

125mg/kg)

C

Kit Control

U1 – U17

Sample

Extracts



11. DETAILED ELISA PROCEDURE

- 11.1. Allow all kit reagents to reach room temperature (18-24°C preferable); prepare and dilute Test Portion extracts & Kit Control, prepare 1X Diluent Solution, as described above.
- 11.2. Ensure that the work area is well organised and tidy, all dilutions are clearly labelled in the correct order (Layout Guide) for pipetting and that ELISA equipment is ready for use; remove caps from all dilutions to speed up addition to the wells.
- 11.3. Mark microwell strips on upper or lower tab (and/or on individual break-apart wells) to keep them in the correct order should they become detached from frame.
- 11.4. Mix the ready to use HRP, TMB and Stop reagents gently just before use.
- 11.5. Quickly but carefully “reverse” pipette, according to the Layout Guide you set out, 100µl of: S0 (Zero), S1–S5 Standards, Kit Control & unknown Extract dilutions into 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 **40 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 times more. After the THREE wash cycles, flick out the plate several times to remove excess solution; tap the wells upside down **FIRMLY** on absorbent paper until little or no liquid appears on the paper and **no bubbles remain**; while inverted, wipe base of wells to clean them.

Alternatively: Use a hand held/automatic plate washer to aspirate then fill wells three times; finally, empty wells then tap onto paper as described above.
- 11.9. Immediately add 100µl of Anti-Fish HRP (blue/green coloured reagent) using a microlitre or repeating pipette; mix as described in 11.6.
- 11.10. Incubate at room temperature for **40 MINUTES**.
- 11.11. Wash all wells FOUR 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. Incubate at room temperature **IN THE DARK** (e.g. in a drawer) for **15 MINUTES** or **until sufficient colour develops (section 16.7; page 15)**.

11.14 - 11.16 on next page...



- 11.14. Add 100µl of Stop Solution 1 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 at least 60 minutes)
- 11.16. Read plate at 450nm using the plate reader and record absorbance values.

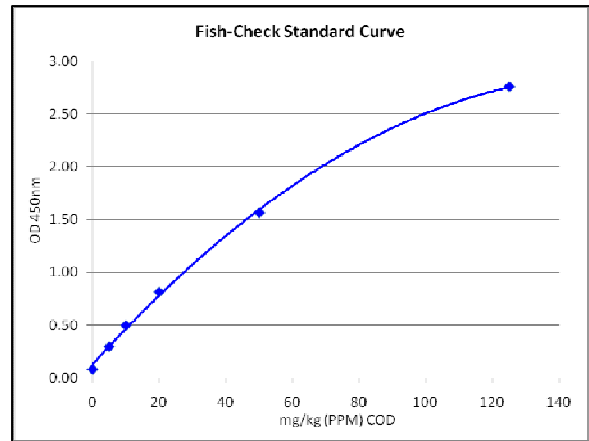
12. CALCULATION OF RESULTS

- 12.1. Plot Standard Curve on graph paper and draw a curve of best fit; read off Kit Control & unknown Sample fish concentrations (mg/kg) and record results on Layout Guide. Alternatively use curve-fit software to produce results.
- 12.2. If the nominal extraction and dilution ratios are NOT used exactly, you **must correct back for the actual weights and volumes used.**

13. EXAMPLE ASSAY DATA

13.1. Example Standard Curve (right)

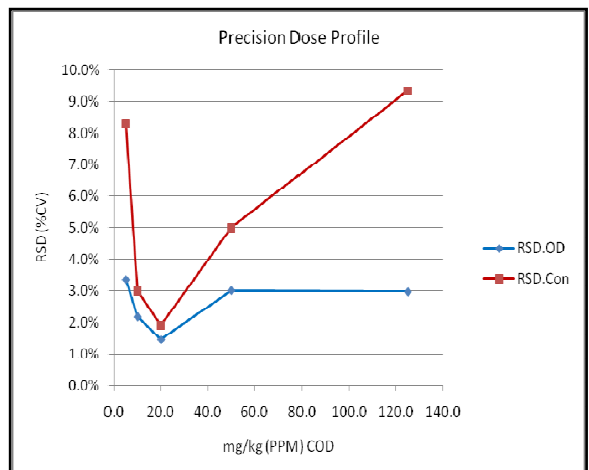
13.1.1.	Zero	0.082 (OD450)
13.1.2.	5mg/kg	0.296
13.1.3.	10mg/kg	0.497
13.1.4.	20mg/kg	0.814
13.1.5.	50mg/kg	1.563
13.1.6.	125mg/kg	2.755



13.2. The Repeatability, limited Reproducibility and Robustness of the method were assessed by performing ten assays with two operators, using two or three lots of all specific reagents both within and at the end of their expiry dates over a period of two months. The data shown in this section is a composite of these ten assays:

13.2.1. Quantitation range

13.2.1.1. The Precision Dose Profile (right) shows a plot of the RSD (%CV) of OD450nm and of Concentration across the Standard Curve. As all Concentration values are below 10% the quantitation range is **5-125 mg/kg (PPM) Cod** or better.





13.2.2. Within Assay RSDs were:

13.2.2.1. **3.0%** for OD450nm (including Zero ODs)

13.2.2.2. **5.8%** for mg/kg Cod (Standards & Kit Control)

13.2.3. Over the ten assays, the Range, Average value and Between Assay RSD (%CV) for the diluted Kit Control were **12.4-14.7mg/kg; 13.2mg/kg & 5.1.**

13.2.4. The Average Limit Of Detection (Zero + 3 x Std. Dev.) was **0.34mg/kg.**

13.2.5. Signal:Noise ratio (5mg/kg Standard:Zero OD averaged **2.7 x** (range 2.6-3.0).

14. INTERPRETATION OF RESULTS

14.1. Assay Calibration. At present there is no agreed calibrator to help support fish detection. The imutest FISH-CHECK ELISA has been calibrated using fresh cod (Class: Actinopterygii; Order: Gadiformes; Family: Gadidae) tissue but estimates of total fish levels using the assay are approximate unless the fish present is known and unprocessed.

14.2. Reactivity: the antibody used in this kit reacts very differently to different fish species:

Monkfish	Tuna	Skate	Mackerel	Salmon	Trout
Actinopterygii	Actinopterygii	Chondrichthyes	Actinopterygii	Actinopterygii	Actinopterygii
Lophiiformes	Perciformes	Elasmobranchii	Perciformes	Salmoniformes	Salmoniformes
Lophiidae	Scombridae	Rajiformes	Scombridae	Salmonidae	Salmonidae
0.01%	0.16%	0.23%	0.74%	8.0%	7.2%

Herring	Sea Bass	Pollack	Coley	Whiting	Haddock
Actinopterygii	Actinopterygii	Actinopterygii	Actinopterygii	Actinopterygii	Actinopterygii
Clupeiformes	Perciformes	Gadiformes	Gadiformes	Gadiformes	Gadiformes
Clupeidae	Moronidae	Gadidae	Gadidae	Gadidae	Gadidae
11.6%	25.0%	31.3%	36.9%	50.4%	56.7%

14.3. Processed food raw materials/products may be significantly less reactive in the assay. Cod muscle gave a cod level of 91% in the assay. Retail, partially-cooked (cod) fish cakes with a labelled content of 40% cod gave a cod level of 28% in the assay. When cod extract was spiked into milk then used to make a (boiled) roux-based white sauce the recovery of fish protein was only ~8%, probably due to heat denaturation of parvalbumin.



14.4. Over 50 individual food commodities have been tested during the development of this assay; all but three were found not to react in the assay. 100% samples of finely ground buckwheat (19ppm), amaranth (8ppm), red lentil (5ppm) & soya (4-5ppm) were found to interfere in the assay, giving false positive results. The commodities tested is listed below:

Alfalfa	Gelatin (porcine)	Peanut
Almond	Gluten, wheat	Pecan nut
Amaranth	Hazelnut	Pine nut
Apricot kernel	Kamut flour	Pistachio
Barley	Lentil (green)	Potato (powder)
Beef, meat	Lentil (red)	Prawn
Brazil nut	Linseed	Pumpkin seed
Buckwheat	Lupin	Quinoa
Cashew	Macadamia	Rice – White; flour
Cheese, cow's	Malt extract	Sesame
Chick peas	Milk (Bovine) powder	Soya flour
Chicken, meat	Milk (Ovine) powder	Spelt flour
Cocoa powder	Millet	Sucrose/sugar
Coconut (flour)	Mung bean	Sunflower
Corn/maize	Mussel	Teff flour
Egg (white)	Mustard	Walnut
Egg (yolk)	Oats	Wheat, flour, white
Gelatin (fish)	Pea	

15. PERFORMANCE INDICATIONS

15.1. Prior to stopping the ELISA, S0 wells should be nearly colourless and there should be a **visible colour difference** between the S0 and pale blue S1 (5mg/kg) wells. The S5 (125mg/kg) wells should be a strong blue colour. Indicative assay parameters are suggested to be as follows:

15.2. Zero OD _{450nm} :	<0.25 units (Preferably <0.20 units).
15.3. Limit of Detection (LOD):	<0.5 PPM (At 3 x Std. Dev. from Zero).
15.4. 5mg/kg Std. OD _{450nm} :	>2.0 x Zero OD.
15.5. 125mg/kg Std. OD _{450nm} :	>1.5 units (Preferably >1.75 units).
15.6. Kit Control:	Refer to C. of A.
15.7. Duplicate precision (RSD OD _{450nm}):	<7.5.
15.8. Duplicate precision (RSD mg/kg cod):	<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. DIL's imutest A6008 & A6009; 100 & 25 swabbing sets respectively) 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 pipettes are kept in good condition & regularly calibrated.
- 16.5. Washing: if more Wash Solution is required e.g. for automatic washers; ELISA automation instruments please contact your supplier for extra supplies.
- 16.6. 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; wipe base of wells to ensure that they are clean and dry.
- 16.7. Consistently attaining ideal levels of colour development (see example data section 13; page 12) depends on:
 - 16.7.1. laboratory temperature; at temperatures below 18°C incubation times tend to be longer and above 24°C they may need to be shortened.
 - 16.7.2. effectiveness of washing; ensure that wells are filled to the rim and remember, it is difficult to over-wash!
 - 16.7.3. 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.
 - 16.7.4. previous experience of this ELISA in your lab.
 - 16.7.5. If you have an OD620nm filter you can monitor colour development after ~12-13 minutes to help predict final (stopped) OD450nm values. These will be ~3 times the predicted OD620nm level at fifteen minutes. Stop the assay when the OD620 value is expected to be between 0.5–0.8 units (depending on reader).
- 16.8. If your plate reader has a pre-mixing facility, set the speed to between 700-900 cycles per minute and time for ~10 seconds before reading.



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 fish 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.

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