Instructions for use
InviLisa® Gluten ELISA Kit

INVITEK diagnostics

InviLisa®

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96 reactions

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1. KIT CONTENTS

COMPONENTS	QUANTITY	READY TO USE
Gluten Diluent (Zero Standard)	4 x 25mL	✓
2.5 mg/kg Gluten Standard (S1)	1 x 1.4mL	Dilute 1:19
10.0 mg/kg Gluten Standard (S2)	1 x 1.4mL	Dilute 1:19
25.0 mg/kg Gluten Standard (S3)	1 x 1.4mL	Dilute 1:19
50.0 mg/kg Gluten Standard (S4)	1 x 1.4mL	Dilute 1:19
LOW Kit Control (<1mg/kg)	1 x 6g	✓
MID Kit Control (See CoA)	1 x 6g	✓
Extraction Solution T	2 x 25mL	Mix and Dilute
Extraction Solution P	2 x 25mL	Mix and Dilute
Wash Solution Concentrate	1 x 55mL	Dilute 1:19
Anti-Gliadin Antibody-Coated Microwell Plate	12 x 8 well strips	✓
Anti-Gliadin HRP Reagent	1 x 12mL	✓
TMB Substrate	1 x 12mL	✓
Stop Solution	1 x 12mL	✓
Document Set	IFU	–

1.1. PREPARATION OF COMPONENTS

Allow refrigerated kit contents to reach room temperature before preparing reagents. If a precipitate/crystals form in Wash Solution or Extraction Buffer concentrates, warm slightly and mix well to re-dissolve before dilution.

Gluten Standards (1X): Prepare by diluting at a ratio of 1:19 (1/20) with assay diluent (e.g. add 50µL concentrate to 950µL assay diluent and mix well). Prepare fresh each time.

Wash Solution (1X): Prepare by diluting at a ratio of 1:19 (1/20) with purified water (e.g. add 10mL concentrate to 190mL water and mix well).

Anti-Gliadin HRP, TMB Substrate and Stop Solution: are all ready to use, no preparation is necessary, simply mix by repeated inversion (do not shake) just before use.

1.2. EXTRACTION SOLUTION

To make 500mL, add 200mL of 100% Ethanol to 250mL of purified water. Then add the supplied 25mL of Extraction T and the 25mL of Extraction P. Carefully mix and allow to cool to room temperature.

Store in a sealed container to avoid evaporation. This will be sufficient for 20-25 samples when using 20mL per extraction.

Lower amount of working extraction solution can be prepared by adjusting the volumes of the corresponding components while keeping the same ratios (⅖ Ethanol, ½ Water, 1⁄20 Extraction T and 1⁄20 Extraction P).

TOTAL VOLUME (mL)	VOLUME ETHANOL (mL)	VOLUME WATER (mL)	VOLUME EXTRACTION T (mL)	VOLUME EXTRACTION P (mL)
500	200	250	25	25
250	100	125	12.5	12.5
200	80	100	10	10
100	40	50	5	5
50	20	25	2.5	2.5

2. STORAGE

All buffers and kit contents of the **InviLisa® Gluten ELISA Kit** should be stored refrigerated (2-8 ºC) and used before their Expiry Dates. Once the kit reagents have been opened, exposure to room temperatures should be minimised.

Before every use, make sure that all components are at room temperature (18-24 ºC). If there are any crystals/precipitates within the provided solutions redissolve these precipitates by placing the bottle in warm water (e.g. in a 37 ºC bath).

Once diluted 1:19, the **Standards** should be used within the day of the Assay. Do not store.

Once diluted 1:19, the **Wash Solution** is stable at room temperature (18-24 ºC) in a sealed clean container for at least two weeks.

Once prepared **Extraction Buffer** is stable at room temperature (18-24 ºC) for one week. Make only enough for one week.

If required for re-testing, test portion extracts can be stored at room temperature (18-24 ºC) and preferably in the dark for at least one week.

3. SAFETY INFORMATION

When and while working with chemicals, always wear a suitable lab coat and avoid skin contact.

For more information, please consult the appropriate material safety data sheets (MSDS). These are available online in convenient and compact PDF format at www.invitek.com for each Invitek Diagnostics product.

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

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

4. INTENDED USE

The InviLisa® Gluten ELISA Kit is designed for quantitation of low levels of Gluten forms from wheat, rye and barley in raw materials, environmental swabs, part-processed and finished food products. This assay utilises monoclonal antibodies to specifically target gliadin with R5-like specificity though with detection of the more toxic deamidated forms. The 96 well kit includes four gluten Standards and has a quantitation range of 2.5 – 60 mg gluten/kg.

5. PRODUCT CHARACTERISTICS

Limit of Detection	**0.1 mg/kg (foods)**
Quantitation Range	**2.5 - 50 mg/kg (foods)** **≥ 16 ng/swab**
Units	**mg gluten/kg**
Specificity	**Gliadin**
Cross-reactivity	**None (from a panel of >80 ingredients)**
Sample Type	**Raw materials, processed foods, surface swabs**
Test Portion	**1g**
Sample Preparation	**Grind, shake, mix, centrifuge**
Time required	**Extraction: 40 min** **ELISA: 20+20+20 min** **Total time: 100 min (10 samples)**
Validation	**Validation followed Best Practices and Guidelines from International Standards such as EN 15482:2019 and EN 15633-1:2019**

The assay utilises the ELISA technique and detects the presence of gluten forms from wheat, rye and barley. The plate microwells are coated with 2D4 monoclonal antibodies which detect gliadin with R5-like specificity though including deamidated forms.

Coeliac disease affects around 1% of the EU, US and Australian populations but is thought to be largely undiagnosed. The disease occurs when cereal gluten triggers damage to the small intestine which can only be managed by adherence to a low gluten diet. The deamidated forms of gluten are toxic to patients and the presence of antibodies to deamidated gliadin peptides is used in the diagnosis of the disease. Codex, EC and US Regulations define gluten-free foods as those containing less than 20 mg gluten/kg.

6. EQUIPMENT & MATERIALS REQUIRED (NOT INCLUDED IN THE KIT)

CONSUMABLES:

- 100% Ethanol to solubilise gluten.
- Purified water for Extraction Buffer and Wash Solution preparation.
- Disposable polypropylene containers and centrifuge tubes for preparing solutions and sample dilutions.
- Swabs and tubes, if collecting environmental samples.
- ELISA plate covers (plastic or adhesive film) to prevent evaporation during incubations.

EQUIPMENT:

- Sample mill, chopper, blender or homogeniser and two place balance (sample preparation, dependent on sample type).
- Heated water bath, set at 60°C. If using a shaking water bath, ensure that it adequately mixes the samples.
- Vortex and rotatory mixers (sample extraction).
- Centrifuge (minimum 1000g, preferably >2000g) capable of spinning 50mL centrifuge tubes.
- Micropipettes and tips (100µL and 1mL).
- Wash bottle (and paper towels) or automated/hand-held ELISA washer for microwell plate washing.
- ELISA plate/strip reader (450nm filter), preferably using ELISA software to calculate results.

7. METHOD OVERVIEW

LABORATORY SAMPLE PREPARATION

- **Prepare** Sample by grinding/chopping/blending until homogeneous.
- **Add** A Test Portion of 1.0g to a 50mL centrifuge tube (record weight).
- **Vortex** For 2 minutes at room temperature* to allow reducing agent to work. Adjust pH.
- **Extract** Incubate for 15 minutes @ 60°C Shaking every 5 minutes for 1 minute.
- **Separate** Centrifuge for 10-15 minutes.
- **Dilute** Sample extracts and Standards 1/20 (1:19) in Gluten diluent.

ELISA PROCEDURE

- **Pipette 100µL** Standards and 100µL diluted Control/Sample extracts into wells.
- **Mix. Incubate** at room temperature for **20 minutes**.
- **Wash wells FIVE** times with Wash Solution.
- **Pipette 100µL** anti-Gluten HRP reagent into wells.
- **Mix. Incubate** at room temperature for **20 minutes**.
- **Wash wells FIVE** 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** mg **gluten**/kg results for all Samples.

IMPORTANT POINTS BEFORE STARTING A PROTOCOL

- Because of the extreme sensitivity of the test, very high standards of cleanliness should be observed when handling samples, using equipment and cleaning down before, between and after all stages in the process.
- Proteins bind strongly to some plastics e.g. polystyrene; it is recommended that new polypropylene or glass containers are used for sample handling.
- To prevent cross-contamination, pipette tips should not be reused.
- “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 e.g. by wiping carefully with clean tissue.
- The assay can be conducted with a single well per extract without compromising the functionality of the test kit. Laboratories may opt for this practice following a careful risk management analysis. However, this does not conform with standards such as EN 15633-1 and EN 15842. It should be noted that this increases the likelihood of errors and results in greater variability.

8. SAMPLING AND PREPARATION OF STARTING MATERIAL AND KIT CONTROLS

8.1. SWAB SAMPLES:

8.1.1. Add 1mL of Extraction & Sample Dilution Buffer (1X) to a polypropylene tube.

8.1.2. Cut off the cotton end of the swab and transfer into the tube.

8.1.3. Vortex for 30-60 seconds.

8.1.4. Swab samples are assayed undiluted (proceed to 9). If high levels are expected, swabbing solutions can be further diluted in Assay diluent if required.

8.2. SOLID AND LIQUID FOOD SAMPLES:

- Finely divided flours/powders, fine breadcrumbs, smooth liquids and Kit Controls require no preparation (proceed to 8.2.1).
- 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.

8.2.1. Weigh out a Test Portion of ~1.0g into the 50mL centrifuge tube.

8.2.2. Add 20mL of prepared Extraction Solution.

8.2.3. Shake vigorously by hand or on a vortex mixer to disperse Test Portion in the Extraction Solution.

8.2.4. Check the pH of the sample, and **adjust to pH 5.0-6.5 if required**.

8.2.5. Place sample into a 60ºC water bath for 15 minutes.

8.2.6. Shake every 5 minutes, for 1 minute, and return to the water bath.

8.2.7. After 15 minutes shake vigorously/vortex and centrifuge at ≥1,000g for 10-15 minutes.

8.2.8. Remove a portion of extract from the liquid layer above the food pellet (food extract) to a clean tube.

8.3. Carefully pipette e.g. 50µL of food extract and add to 950µL of Assay Diluent (a 1/20 dilution). Mix well.

NOTE: When using the above weights/volumes, the 5-point standard curve equates to 2.5-50 mg gluten/kg.

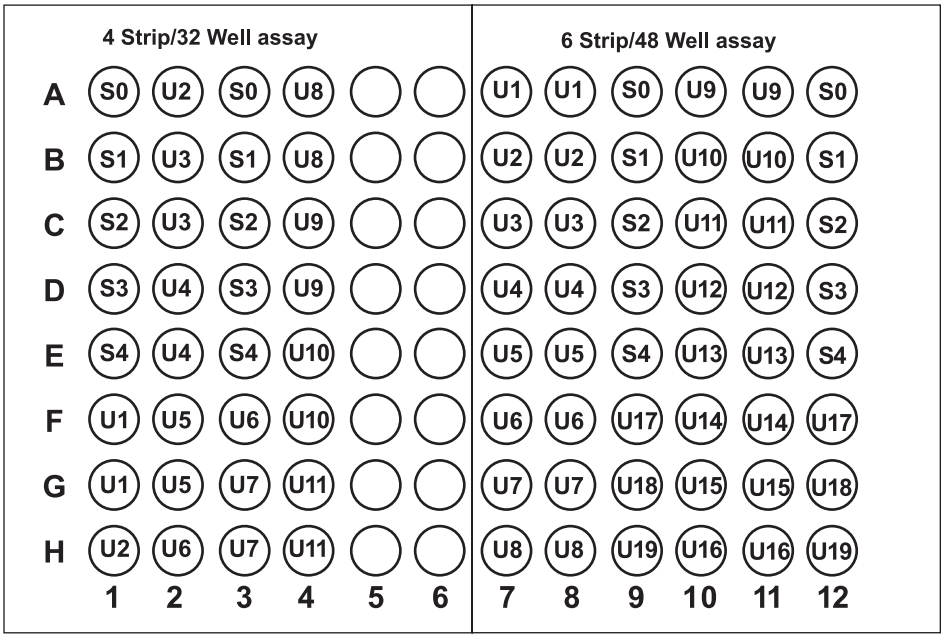
For foods expected to contain higher levels of gluten, dilute the extract by e.g. a further:

- 5-fold 5-point standard curve equates to 12.5 to 250 mg gluten/kg.
- 20-fold 5-point standard curve equates to 50 to 1,000 mg gluten/kg.

9. ELISA PROCEDURE

9.1. Allow kit reagents to reach room temperature (18-24 ºC); prepare reagents and Test Portion extracts, diluted, if necessary, as described above.

9.2. Suggested Quantitative Assay Layouts for 5-point standard curve (32- & 48-well assays) are shown in the figure below.



Key to Layout:

S0 – S4

Gluten Standards (Zero-50 mg/kg)

U1 – U19

Kit Controls

Sample Extracts

9.3. Ensure that the work area is well organized and tidy, all extracts are clearly labelled in the correct order (Layout Guide) for pipetting and that ELISA equipment is ready for use.

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

9.5. Remove caps from all diluted Standards/extracts/dilutions to speed up pipetting.

9.6. Mix the HRP Conjugate, TMB and STOP reagents gently just before use.

9.7. Add 100µL of each Standard, Kit Control and Diluted Sample Extract to the appropriate well using a microlitre pipette.

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

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

9.10. 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 four times more. After the **FIVE** 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.

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

9.12. Immediately add 100µL of Anti-Gliadin HRP reagent using a microlitre or repeating pipette; mix as described in 9.8.

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

9.14. Wash all wells **FIVE** times with 1x Wash Solution as in 9.10.

9.15. Immediately add 100µL of TMB Substrate to all wells; mix as described in 9.8.

9.16. Cover plate; incubate at room temperature for **20 minutes IN THE DARK** (e.g. in a drawer).

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

9.18. Mix plate as described in 9.8 to stop enzyme activity and evenly distribute colour.

9.19. Colour remains stable for up to 15 minutes.

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

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

10. CALCULATION OF RESULTS

10.1. Prepare a Standard Curve by plotting e.g. mg gluten/kg against Standard mean OD. Use curve-fit software using a 4PL (four parameter logistic regression) curve to produce the results. Record results on Layout Guide.

- Alternatively use normal graph paper. Draw a line/curve of best fit and, using their OD values, read off unknown Sample concentrations from the curve.

10.2. The gluten content (mg/kg) of each sample can only be read directly from the standard curve when the nominal extraction procedure is followed i.e. dilution equals 1/20.

IMPORTANT NOTE: If the nominal extraction and dilution ratios are NOT used, any differences must be taken into account by correcting back for the actual weights and volumes used.

10.3. For swab samples, the approximate estimates of the gluten content are taken from the Standard Curve as follows: a swab sample (2.5mL, undiluted) giving an absorbance which interpolates at 2.5 mg/kg on the Standard Curve contains:

2.5/400 = 0.00625 mg gluten/L (or 6.25ng/mL) x 2.5 = ~16 ng/swab

10.4. Whilst this 400-fold factor and conversion to ng/swab could be applied to any undiluted swab sample that gives an absorbance value falling within the quantitation range, swab results are best reported as qualitative (< or > 16 ng gluten/swab).

11. INTERPRETATION OF RESULTS

11.1. Reactivity: InviLisa® Gluten ELISA Kit is calibrated using PWG Reference Material and (FAPAS) samples. 2D4 antibodies detect cereal gliadins with R5-like specificity and glutamine residue forms that have undergone deamidation, these deamidated forms of gluten are highly potent to sensitive individuals.

11.2. Effects of processing: deamidation of gluten can occur when foods are processed, with a combination of heating and acidification. Deamidation can also occur intentionally in the production of some wheat protein isolates, which are used for a diverse range of functions in foods and drinks e.g. emulsifiers, gelling/improving/clarifying agents. Although the level of deamidation does vary, their potential presence in foods and drinks together with their potent nature means it is important for those with coeliac disease and food hypersensitivities to determine both unmodified and deamidated forms of gluten.

11.3. Recovery after spiking into seven matrices was as follows:

- Bread mix **90%**
- Buckwheat pancake mix **97%**
- Skimmed milk powder **95%**
- Gravy powder **92%**
- Vegan sausage **102%**
- Dark chocolate **95%**
- Rice flour **91%**
- Gluten free oats **85%**
- Soup mix **104%**
- Cumin **93%**
- Beef flavour mix **82%**
- Dry biscuit **90%**
- Cream biscuit **91%**
- Pasta **95%**
- Vegan margarine **92%**
- Chocolate ice cream **87%**

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

Adzuki beans	Coconut (flour)	Macadamia	Quinoa (flour)
Almonds	Coffee beans	Milk (fresh, full cream)	Red kidney beans
Amaranth	Coriander (seeds)	Millet	Rice (brown)
Apricot (dried)	Corn / Maize (cob)	Mung bean	Rice (white)
Balsamic Vinegar	Corn / Maize (flour)	Mustard (brown)	Rice (red)
Basil (fresh)	Cumin (seeds)	Mustard (yellow)	Rosemary (fresh)
Beef	Dates	Oats (gluten free)	Sesame seed
Black beans	Egg (whole)	Olives (black)	Skimmed milk powder
Brazil nuts	Fennel (seeds)	Paprika (sweet)	Sorghum (flour)
Broccoli	Fish (Basa, raw)	Pea protein (powder)	Soya bean
Buckwheat	Flaxseed	Peanuts	Soya milk
Cabbage	Garlic powder	Pecans	Split peas (green)
Calamari	Green banana flour	Peppercorn (black)	Sugar (white)
Cashew nut	Green banana flour	Pine nut	Sunflower seeds
Celery seed	Green banana flour	Pistachio	Tapioca (flour)
Chestnut flour	Green banana flour	Poppy seeds	Tea (Earl Grey)
Chia seeds	Lecithin (soy)	Pork (raw)	Teff (grain)
Chicken (raw)	Lentils (whole, green)	Potato starch	Tomato paste
Chickpea	Lima beans	Prawn (Green Banana)	Walnut
Chilli powder	Lupin seed (L. albus)	Pumpkin seed	Wine (red)
Cocoa	Lupin seed (L. angustifolius)	Psyllium	Wine (white)

IMPORTANT NOTE: Only the above food commodities have been tested for potential cross reactivity; it should be assumed that commodities not on this list may react in the assay and they should be appropriately validated. Please bear in mind the need for testing only 100% authentic commodities to determine possible cross reactivity.

12. PERFORMANCE INDICATIONS

Prior to stopping the ELISA, S0 wells should be nearly colourless and there should be a slight colour difference between the S0 and pale blue S1 (2.5 mg/kg) wells. The S4 (50.0 mg/kg) wells should be a mid-blue colour. Indicative assay parameters are suggested to be as follows:

Zero OD_{450nm}	<0.15 units
Limit of Detection (at 3 x Std. Dev. from Zero)	0.1 mg gluten/kg
2.5 mg gluten/kg OD_{450nm}	>3 x Zero OD_{450nm}
25 mg gluten/kg OD_{450nm}	>1.0 unit; preferably >1.25 units
50 mg gluten/kg OD_{450nm}	>1.5 units; preferably >1.75 units
Kit Control values	Refer to Certificate Of Analysis
Duplicate precision (mg/kg)	<10 RSD
Duplicate precision (OD_{450nm})	<10 RSD

Please refer to the kit's CoA for the Standard Curve data representative of the batch.

A Validation Report is available from Invitek Diagnostics which summarises the findings in our laboratories with respect to e.g. sensitivity, specificity, repeatability, reproducibility, robustness.

13. TROUBLESHOOTING

Assay parameters indicative of VALID performance are as follows:

PROBLEM	POSSIBLE REASON	CORRECTIVE ACTION
Poor duplicates	(i) Poorly maintained pipettes (ii) Contamination (iii) Inadequate / inconsistent plate washing	(i) Ensure pipettes are kept in good condition, regularly serviced and calibrated. (ii) Avoid splashing and contamination of ready to use reagents. (iii) Ensure wells are filled to the rim, it is difficult to over-wash; if using a wash bottle flick out well contents vigorously; avoid bubbles during the last wash by carefully overfilling when using a wash bottle or aspirate away when using a manual washer; 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 they are clean and dry.
High background	(i) Inadequate / inconsistent plate washing (ii) Contamination	(i) See above advice (ii) Good laboratory practice reduces the possibility of cross contamination; validate laboratory/ equipment cleaning regimes to ensure very high standards of cleanliness.
Assay drift	(i) Interrupted set-up (ii) Reagents not at room temperature	(i) Ensure that all samples, standards and controls are prepared appropriately before starting the assay to ensure the assay is performed continuously. (ii) Ensure that all reagents are at room temperature (18 - 24 ºC) before pipetting into wells.
Low or flat Standard Curve	(i) Reagents not at room temperature (ii) Incorrect procedure	(i) See advice above (ii) Refer to CoA; check procedure used (including reader) and eliminate modifications, if any.
Response too high (high ODs)	(i) Room temperature too high (ii) Contamination	(i) Adjust room temperature or by monitoring colour development to fit to the range of the reader used. (ii) See advice above.

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