Instructions for use
InviLisa® Casein ELISA Kit

INVITEK diagnostics

InviLisa®

Language: EN

RUO

REF 6032001200

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ALS Life Sciences Portugal, S.A.
Zona Industrial de Tondela, ZIM II,
Lote 6, 3460-070 Tondela
Portugal

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TABLE OF CONTENT

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1. KIT CONTENTS

COMPONENTS	QUANTITY	READY TO USE
0 mg casein/kg Casein Standard (S0)	**1 x 1.4mL**	✓
0.2 mg casein/kg Casein Standard (S1) (equivalent to 0.65 mg SMP/kg)	**1 x 1.4mL**	✓
1.0 mg casein/kg Casein Standard (S2) (equivalent to 3.2 mg SMP/kg)	**1 x 1.4mL**	✓
2.5 mg casein/kg Casein Standard (S3) (equivalent to 8.0 mg SMP/kg)	**1 x 1.4mL**	✓
5.0 mg casein/kg Casein Standard (S4) (equivalent to 16.0 mg SMP/kg)	**1 x 1.4mL**	✓
Extraction & Sample Dilution Buffer	**2 x 25mL**	**Dilute 1:19**
Washing Solution Concentrate	**1 x 55mL**	**Dilute 1:19**
Anti-Casein Antibody-Coated Microwell Plate	**12 x 8 well strips**	✓
Anti-Casein HRP Reagent	**1 x 12mL**	✓
TMB Substrate	**1 x 12mL**	✓
Stop Solution	**1 x 12mL**	✓
Document Set	**IFU**	–

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.

Extraction & Sample Dilution Buffer (1X): Prepare by diluting at a ratio of 1:19 (1/20) with purified water (e.g. add 5mL concentrate to 95mL water and mix well).

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).

Casein Standards, Anti-Casein 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.

2. STORAGE

All buffers and kit contents of the **InviLisa® Casein 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 **Wash Solution** is stable at room temperature (18-24 ºC) in a sealed clean container for at least two weeks.

Once diluted 1:19 the **Extraction & Sample Dilution Buffer** is stable at room temperature (18-24 ºC) for one week.

If required for re-testing, test portion extracts can be stored frozen at or below -18 °C; they remain stable for several weeks.

3. INTENDED USE

The InviLisa® Casein ELISA Kit is designed for quantitation of low levels of casein in various food and beverage matrices, including baked goods, beverages, cereals/cereal products, confectionery, meat products, and wine, as well as environmental swabs. This assay utilises polyclonal antibodies to specifically target casein, which is the most abundant protein in milk, as a marker for the presence of milk, caseins and caseinates. The 96 well kit includes four casein Standards and has a quantitation range of ~0.2–5 mg casein/kg.

4. 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.

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5. PRODUCT CHARACTERISTICS

Limit of Detection	**0.05 mg/kg (foods)**
Quantitation Range	**0.2 - 5 mg/kg (foods)** **≥ 25 ng/swab**
Units	**mg casein/kg**
Specificity	**Casein**
Cross-reactivity	**Sheep milk at a level of 0.09%** **Goat milk at a level of 0.2%**
Sample Type	**Raw materials, processed foods, surface swabs**
Test Portion	**0.5g**
Sample Preparation	**Grind, chop, blend, heat, centrifuge**
Time required	**Extraction: 40 min** **ELISA: 20+20+20 min** **Total time: 120 min (10 samples)**
Validation	**Validation followed Best Practices and Guidelines from International Standards such as EN 15482:2019 and EN 15633-1:2019**

This assay utilises polyclonal antibodies to detect casein, as a marker for the presence of milk, caseins and caseinates, using a direct “sandwich” ELISA technique. It is designed to detect caseins at low levels in food raw materials, fresh/lightly processed food products and wine as well as in environmental swabs.The assay range is nominally between ~0.20 - 5.0 mg casein/kg.

Skim Milk Powder (SMP) contains ~30% Casein, giving an assay range of ~0.65 - 16.0 mg SMP/kg content, if the stated extraction ratio is used and the extract is not diluted. The Limit Of Detection (LOD) of the assay is ~0.05 mg casein/kg, which corresponds to ~0.2mg SMP/kg content. The range can be extended upwards if required by increasing the extraction ratio and/or diluting the sample extract before testing.

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

CONSUMABLES:

- Purified water for Extraction & Sample Dilution Buffer and Wash Solution preparation.
- Disposable polypropylene containers and centrifuge tubes for preparing solutions and sample dilutions.
- Swabs and tubes, if collecting environmental samples.
- Tannin Binding Additive for use when extracting samples containing polyphenols (e.g. chocolate).
- 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.

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7. METHOD OVERVIEW

LABORATORY SAMPLE PREPARATION

- **Prepare** Sample by grinding/chopping/blending until homogeneous.
- **Add** 1 part Test Portion to 20 parts Extraction & Sample Dilution Buffer 1X.
- **Extract** Incubate for 15 minutes @ 60°C Shaking every two minutes.
- **Separate** Centrifuge for 10 minutes.

ELISA PROCEDURE

- **Pipette 100µL** Standards and 100µL Sample Extracts into wells.
- **Mix. Incubate** at room temperature for **20 minutes**.
- **Wash wells FIVE** times with Wash Solution.
- **Pipette 100µL** anti-casein 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 **casein**/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

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).

8.2. LIQUID SAMPLES:

8.2.1. Add e.g. 0.5mL of homogeneous sample to 9.5mL of the Extraction & Sample Dilution Buffer (1X). For diluted wine samples, check pH and adjust, if necessary, to pH 7.0.

8.2.2. Centrifugation is not normally necessary if the mixture is clear, otherwise centrifuge for 10 minutes at 2000g. Proceed to ELISA procedure (9).

8.3. SOLID FOOD SAMPLES:

8.3.1. Finely divided flours/powders and fine breadcrumbs require no preparation (proceed to 8.3.3).

8.3.2. Non-homogeneous samples e.g. sausages, meat products are prepared by taking a representative portion of the sample and preparing by milling, grinding, chopping, blending etc. until a fine particle size/homogeneity is achieved.

8.3.3. Weigh out a Test Portion of 0.5g of each sample into e.g. a polypropylene tube/ universal container.

8.3.4. Record exact weight added – you do not have to add exactly 0.5g, but should record the weight and correct for the weight used when calculating results.

8.3.5. Add 10.0mL of Extraction & Sample Dilution Buffer (1X). Shake well/vortex or otherwise mix the Test Portion with the Extraction solution.

8.4. Place the sample into a pre-heated water bath at 60°C for 15 minutes shaking every 2-3 minutes to aid allergen extraction.

8.5. Pipette a portion of the extract into a suitable centrifuge tube or, if possible, spin the whole tube at ≥2,000g for 10 minutes and take from the resulting supernatant.

8.6. Alternatively, allow to settle for at least 30 minutes or until a reasonably clear layer appears above the settled food.

8.6.1. If a fatty layer appears above the extraction solution it is best to take from below the fat layer with minimal disturbance.

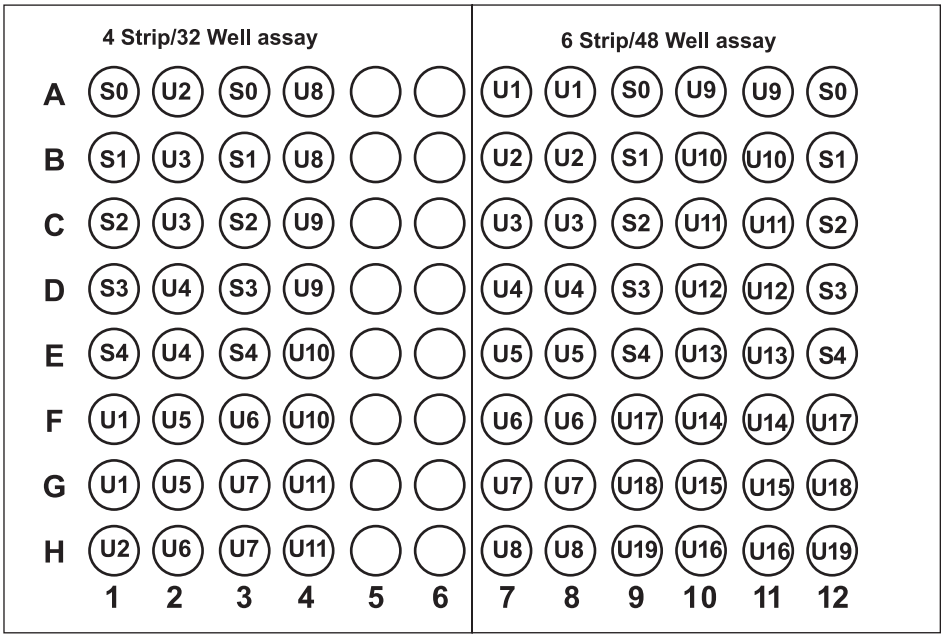
8.7. If further dilution is required for any other extracted samples, dilute with Extraction Buffer 1X.

8.8. If any extracts are diluted after the 1/10 extraction step, ensure that results are corrected for the increased dilution ratio before reporting allergen levels.

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

Casein Standards (Zero-5 mg/kg)

U1 – U19

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 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 and Sample Extract (diluted if necessary) 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-Casein 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 casein/kg against Standard OD values. 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. For simplicity, the standard curve below plots OD_{450nm} against mg casein/kg.

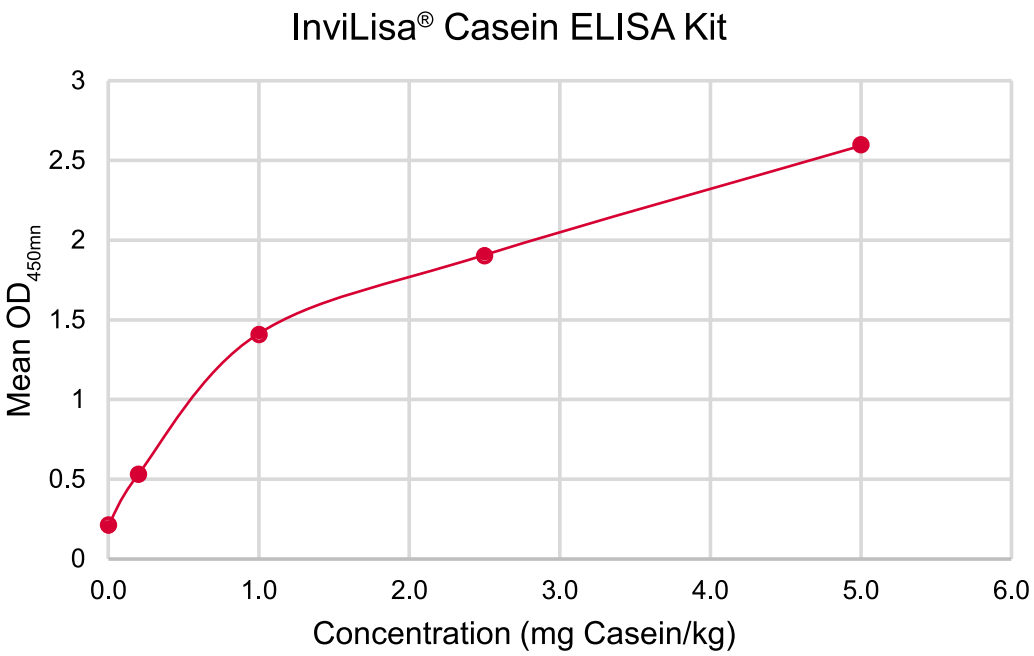
10.3. If preferred, Standard ODs can be plotted against SMP values (0; 0.65; 3.2; 8.0; 16.0 mg/kg) in order to determine SMP values by reading unknown ODs off the standard curve.

IMPORTANT NOTE: During the calculation process, remember, if necessary, to correct for the actual weights used for each sample and any dilutions made post-extraction.

EXAMPLE ASSAY DATA

Zero Standard:	OD_{450nm} 0.199
0.20 mg casein/kg	OD_{450nm} 0.530
1.00 mg casein/kg	OD_{450nm} 1.419
2.50 mg casein/kg	OD_{450nm} 1.914
5.00 mg casein/kg	OD_{450nm} 2.599

Example InviLisa® Casein ELISA Kit Standard Curve:



11. INTERPRETATION OF RESULTS

11.1. The casein content of cow's milk is fairly constant, but the reactivity of commercial casein preparations varies; if the presence of caseins/caseinates is suspected, unless the material of origin is known, it may not be possible to determine any more than the (mg/kg) casein content of the material under test.

11.2. Assay Calibration: at present there is no agreed calibrator to help support Milk allergen analysis. The InviLisa® Casein ELISA Kit has been calibrated using a panel of milk free/low milk content spiked food samples.

11.3. Recovery after spiking with casein into nine matrices was as follows:

- Bread crumbs **92%**
- Cookies **83%**
- Dark Chocolate + additive **80%**
- Orange juice **107%**
- Sausage **84%**
- Soya Drink **98%**
- Red wine + additive **83%**
- Rosé wine **81%**
- White wine **105%**

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

Almond	Corn	Oats	Soya milk
ß-Lactoglobulin (bovine)	Dried cranberries	Orange	Soya peptone
Barley	Egg	Peanut	Sucrose
Beef meat (raw)	Garlic	Pecan Nut	Sunflower Seeds
Brazil nut	Gelatin (Pork)	Pork meat (raw)	Sultanas
Cocoa	Gluten	Pistachio Nut	Tiger nut milk
Cashew milk	Hazelnut Milk	Pumpkin Seed	Wheat
Chicken (raw)	Macadamia Nut	Rice	Wine (white, red, rosé)
Chestnut	Mahaleb Kernal	Rye	
Chickpea	Mustard	Sesame	
Coconut drink	Oat drink	Soya	

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 (0.20 mg casein/kg) wells. The S4 (5.0 mg casein/kg) wells should be a mid-blue colour. Indicative assay parameters are suggested to be as follows:

Zero OD_{450nm}	<0.25 units
Limit of Detection (at 3 x Std. Dev. from Zero)	<0.1 mg casein/kg
0.20 mg casein/kg OD_{450nm}	>1.5 x Zero OD_{450nm}
5.0 mg casein/kg OD_{450nm}	>1.0 unit; preferably >1.5 units
Duplicate precision (OD_{450nm})	Ideally <5%
Duplicate precision (mg/kg)	Ideally <10 – 15%

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 by monitoring colour development to fit to the range of the reader used. (ii) See advice above.

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PORTUGAL
Zona Industrial de Tondela, ZIM II, Lote 2 e 6
3460-070 Tondela
Portugal

Phone: +351 232 817 817

GERMANY
Robert-Rössle-Str. 10
13125 Berlin
Germany

Phone: +49 30 9489 2908

info@invitek.com
www.invitek.com

DE 558.03
28-01-2025