GLUTEN-TEC® ELISA

(5171GT[10]03.20)

Competitive enzyme immunoassay
for the quantitative determination of gliadin
and gliadin fragments in food

EUROPROXIMA GLUTEN-TEC® ELISA

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TABLE OF CONTENTS

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BRIEF INFORMATION

The Gluten-Tec® ELISA is a competitive enzyme immunoassay for the quantitative detection of gliadin and gliadin fragments in food. Samples and standards are measured in duplicate which means that a total of 40 samples can be analysed with one kit.
The Gluten-Tec® ELISA contains all the required reagents, including standards, to perform the test. No reagents for sample preparation are included.

1. INTRODUCTION

Celiac disease (CD) is an autoimmune disease leading to damage of the small intestine. It is caused by the reaction to gliadin, a component of gluten present in wheat and other crops such as barley and rye. The Leiden University Medical Center (LUMC) developed and characterized monoclonal antibodies directed against gluten-derived T cell immune-stimulatory epitopes (toxic epitopes) involved in CD. The anti-α20 gliadin antibody was selected to develop a sensitive competitive ELISA that detects a well-characterized toxic epitope in wheat, barley, rye and their crossbred varieties.

The Leiden University Medical Center, in cooperation with EuroProxima, has developed Gluten-Tec®, a novel competitive ELISA, which detects a well characterized T cell stimulatory epitope of gliadin and homologue sequences present in wheat, barley, rye and their crossbred varieties. Synthetic peptides are used for calibration, which allows an accurate and reproducible standardization. The assay can detect not only intact but also hydrolyzed proteins.

The monoclonal antibody and peptide sequence are protected by patents. The EU patent is granted under EP1779115.

2. PRINCIPLE OF THE GLUTEN-TEC® ELISA

α20 gliadin peptide standards or samples are added to the wells of the ELISA plate coated with anti-α20 antibody. Then α20 gliadin peptide-HRP conjugate is added to each well and the plate is incubated for 3 hours at 4°C. After the washing step the amount of bound conjugate is visualized by addition of TMB substrate. The reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at 450 nm.

3. SPECIFICITY AND SENSITIVITY

The monoclonal antibody used in the test is specific for a T cell stimulatory peptide on the gliadin molecule from wheat and related prolamins from rye and barley. These epitopes play a dominant role in triggering of celiac disease. No cross-reaction is observed with oats, corn, rice, millet and buckwheat.
The test results are expressed in gliadin equivalents calculated from the peptide standard curve.

Sensitivity of the test was determined in oats.

Oats (blank)	gliadin mg/l (ppm)
LOD	2.9
LOQ	3.6

Recoveries were determined in oats spiked with PWG-gliadin standard, wheat, rye and barley at two different levels: 5 ppm and 10 ppm.

	% Recovery	
Oats spiked with:	5 ppm spike	10 ppm spike
PWG-gliadin standard	108	96
Wheat	108	111
Rye	112	97
Barley	125	111

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored in a refrigerator (2°C to 8°C) before and immediately after use.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate at ambient (room) temperature before use.
- Avoid condensation in the wells of the plate. Bring the sealed plate at ambient temperature before opening the plate sealing.
- Exposure of the TMB substrate to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

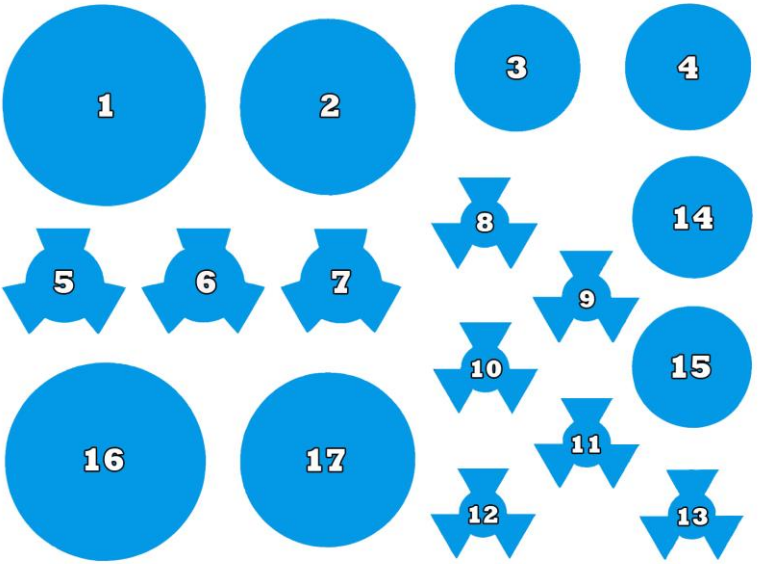
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with α20 antibody. Ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Dilution buffer** (20 ml, 4x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **TMB substrate** (12 ml, ready-to-use)
4. **Stop solution** (12 ml, ready-to-use)
5. Not in use
6. Not in use
7. Not in use
8. **Conjugate Peptide-HRP** (0.1 ml, 100x concentrated)
9. Not in use
10. Not in use
11. Not in use
12. Not in use
13. **Zero Standard** (2 ml, ready-to-use)
14. **Standard solution 1** (1 ml, ready-to-use) **0.156 ng/ml alpha-20-peptide**
15. **Standard solution 2** (1 ml, ready-to-use) **0.313 ng/ml alpha-20-peptide**
16. **Standard solution 3** (1 ml, ready-to-use) **0.625 ng/ml alpha-20-peptide**
17. **Standard solution 4** (1 ml, ready-to-use) **1.25 ng/ml alpha-20-peptide**
18. **Standard solution 5** (1 ml, ready-to-use) **2.5 ng/ml alpha-20-peptide**
19. **Standard solution 6** (1 ml, ready-to-use) **5 ng/ml alpha-20-peptide**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Greiner 15 ml screw cap vial
- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser
- Vortex mixer
- Head-over-head shaker
- Centrifuge (2000 x g)
- Automated microtitre plate washer or 8-channel micropipette 100 – 300 µl
- Microtitre plate shaker
- Microtitre plate reader with 450 nm filter
- Micropipettes, 10 – 1000 µl
- Multipipette with 2.5 ml combitips
- Hot plate magnetic stirrer
- Distilled water
- Ethanol absolute, ≥99.5% (v/v)
- Fish gelatin (Sigma G7765)

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

8. SAMPLE PREPARATION

Due to the nature of the samples analysed (homogenized cereals and their products) cross-contamination between the samples and also contamination from the laboratory environment can occur very easily. Special precautions should be taken to prevent this:

- gloves and lab coats should be worn all the time while performing the test
- surfaces and equipment used should be cleaned with 60 % ethanol.

8.1 Food commodities with the exception of products containing buck wheat, chocolate, coffee, cocoa or tannin

- weigh 0.5 g of a representative homogenized sample and add 4.5 ml 60% ethanol solution (chapter 9)*
- shake head over head for 20 minutes at 60°C
- centrifuge: 10 minutes at 2000 x g at 20ºC to 25ºC
- dilute the supernatant 1:10 with dilution buffer (i.e. 50 µl supernatant + 450 µl dilution buffer)
- use 50 µl per well in the assay.

8.2 Products containing buck wheat, chocolate, coffee, cocoa or tannin

- prepare fish gelatin solution (chapter 9)**
- weigh 0.5 g of homogenized sample into a 15 ml Greiner screw cap vial
- add 4.5 ml of fish gelatin solution (fish gelatin buffer precipitates, stir the solution during pipetting), vortex for 30 seconds
- mix head over head for 30 minutes
- centrifuge 10 minutes at 2000 x g at 20ºC to 25ºC
- dilute the supernatant 1:10 with dilution buffer (i.e. 50 µl supernatant + 450 µl dilution buffer)
- use 50 µl per well in the assay

8.3 Beer and liquid food commodities

- add to 0.5 ml of a liquid sample 4.5 ml 60% ethanol solution (chapter 9)*
- shake head over head for 20 minutes at 60°C
- centrifuge: 10 minutes at 2000 x g at 20ºC to 25ºC
- dilute the supernatant 1:10 with dilution buffer (i.e. 50 µl supernatant + 450 µl dilution buffer)
- use 50 µl per well in the assay.

9. PREPARATION OF REAGENTS

Before starting the assay, reagents should be brought up to ambient temperature (20°C - 30°C). Any reagents not used should be put back into storage immediately at 2°C to 8°C.

Prepare reagents freshly before use

Microtiter plate
Bring the plate to ambient temperature before opening to avoid condensation in the wells. Return unused strips into the resealable bag with desiccant and store at 2°C to 8°C for use in subsequent assays. Retain also the strip holder.

Rinsing buffer
The rinsing buffer is 20 times concentrated. Prepare fresh dilution before use. For each strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Dilution buffer
The dilution buffer is 4 times concentrated. The concentrated buffer should be brought to room temperature and thoroughly mixed before dilution (20 ml buffer + 60 ml distilled water). Concentrated buffer can show precipitates. Mix well before dilution with distilled water. The 4 times diluted buffer can be stored in a refrigerator (2°C to 8°C) until the expiry date stated on the kit label.

Conjugate peptide-HRP (100 µl)
The conjugate (Peptide-HRP) is 100 times concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min, 1000 x g). Add 10 µl of the concentrated conjugate solution to 990 µl of dilution buffer. Per 2 x 8 wells 0.8 ml of diluted conjugate is required. Store unused concentrated conjugate at +2ºC to +8ºC.

TMB Substrate
The TMB substrate (ready-to-use) tends to precipitate at +4ºC. Make sure that this vial is at room temperature before use (keep in the dark) and mix the content before pipetting into the wells.

* 60% Ethanol solution
Add 200 ml distilled water to 300 ml ethanol (absolute) and mix well.

** Fish gelatin solution
Add to wide neck glass bottle:
Add 100 ml distilled water, 300 ml ethanol (absolute) and 25 g of fish gelatin to a wide neck glass bottle. Adjust the volume to 500 mL with distilled water. Close bottle with the cap and heat (rotating) to 60ºC till the solution is homogeneous and transparent. Store for maximum one week at room temperature.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual rinsing solution from the wells.
6. Do not allow the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, make sure that all wells can be aspirated completely and that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).

2. Pipette 100 µl of the zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the zero standard in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.156, 0.313, 0.625, 1.25, 2.5 and 5 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).

4. Pipette 50 µl of conjugate (Peptide-HRP) into all wells, except the blank H1 and H2.

5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate for 3 hours in the dark at 4°C

7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

8. Pipette 100 µl of substrate solution into each well.

9. Incubate 30 minutes at 20ºC to 25ºC in the dark.

10. Add 100 µl of stop solution into each well.

11. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard (Bmax, wells A1 and A2) and multiplied by 100. The zero standard (Bmax) is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard (Bmax)}} \times 100 = \% \text{ maximal absorbance}$$

Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

Alternative for calibration curve:
The absorbance value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The scale of the Y-axis is logit and the X-axis is logarithmic.

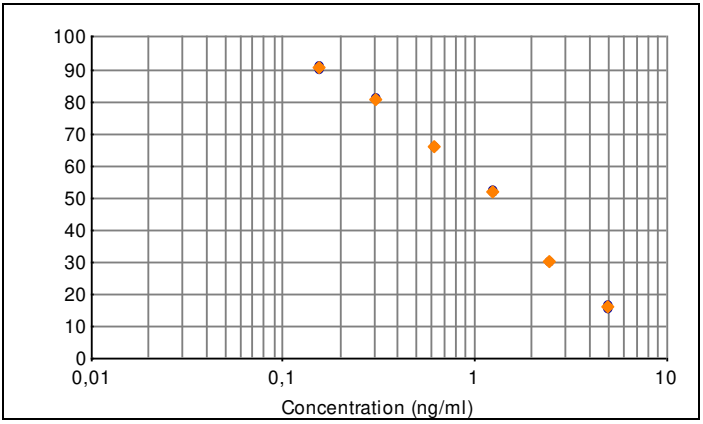


Figure 1 : Example of a calibration curve

To obtain the alpha-20-peptide concentration in a sample (ng/g; ppb), the alpha-20-peptide concentration read from the calibration curve has to be multiplied by a factor 100. The amount of peptide in the samples is correlated to the gliadin content.
gliadin concentration = alpha-20 peptide concentration × 250
gluten concentration = gliadin concentration × 2

12. LITERATURE

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13. ORDERING INFORMATION

For ordering the GLUTEN-TEC® ELISA kit, please use cat. code 5171GT.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

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