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SENSISpec INgezim
GLUTEN R5

Prod Ref: 30.GLU.K2

Double antibody sandwich immunoenzymatic assay for quantitative analysis of gluten in food samples.

Last revision: 05-06-2020

PERFORMANCE TESTED
AOAC
RESEARCH INSTITUTE
LICENSE NUMBER 052005

KIT COMPOSITION

Reagent	1 plate box (8x12 wells)	
	Uni.	Vol.
96 well microtitration plates (12x8 wells) coated with the R5 Mab, specific for gliadin, secalin, and hordein	1	
Positive control ready to use	1	1.5 mL
Negative control ready to use	1	1.5 mL
Control point (20 x concentrated)	1	250 µL
Vials with Gliadin European Standard containing solutions of 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.12 ng/mL and 1.56 ng/mL ready to use	5	1.5 mL
Vials with an anti-gliadin, secalin and hordein R5 Mab-peroxidase conjugate (ready to use)	1	15 mL
Bottle with extraction buffer (ready to use)	1	110 mL
Bottle with Washing solution 25x concentrated	1	65 mL
Bottle with Diluent (DE12-05) (5x concentrated)	1	65 mL
Bottle with TMB Substrate (ready to use)	1	15 mL
Bottle with Stop Solution (H_2SO_4 0,5M) (ready to use)	1	15 mL

OTHER MATERIALS AND REAGENTS NEEDED NOT PROVIDED WITH THE KIT:
Ethanol Distilled water

The SENSISpec INgezim Gluten R5 kit has been granted AOAC Research Institute *Performance Tested Methods*^{SM} status, and assigned certification number #052005, for gluten-free bread mix, oat flour and stainless steel surfaces.

TECHNICAL BASIS

This kit is based on a double antibody sandwich enzyme immunoassay (DAS or capture Elisa). We make a brief description of the technique below:
The plate is coated with the R5 monoclonal antibody (Mab), which recognises an epitope common to gliadins, secalins and hordeins. Therefore, when a sample is added into the wells, the capture R5 Mabs will bind the gliadins present in it. A second R5 Mab, conjugated with Peroxidase is added after a washing step.

This conjugated Mab will bind to the captured gliadins and after a washing step the peroxidase action will be detected by adding the TMB substrate. Change of the colourless substrate solution into a blue product and then, after addition of stop solution, into a yellow colour can be measured with an ELISA reader.
Gliadin content of the samples will be determined by extrapolation of their OD in the calibration curve done with the gliadin standard supplied with the kit. The sensitivity of the assay is 3 ppm of gluten.

PRECAUTIONS AND WARNINGS FOR USERS:

Read the use instructions carefully.
Bring all reagents to room temperature (22°- 25°C) prior to use.
Do not mix instructions or reagents from different kits.
Avoid any contamination of the reagents of the Kit.
Do not use components after expiration dates and do not mix components from different lots.
There should be no eating, drinking, or smoking where specimens or Kit reagents are being handled.
Do not pipette by mouth.
Use a new tip for each new sample.
Include the controls and the standard curve every time the assay is run.
IMPORTANT TMB Substrate must be handled whit care, it is very sensitive to light and contamination: pipette a sufficient amount for the assay from the substrate storage bottle into a separate dark container prior to colour develop step.
Stop solution is a strong acid. Handle with care

STORAGE OF THE KIT COMPONENTS:

All components must be stored between +2ºC and +8ºC. DO NOT FREEZE ANY OF THE KIT COMPONENTS.
Avoid exposure of the kit and the components to direct sunlight at any time, as some reagents are light sensitive.

INFORMATION ABOUT THE WASHING STEPS:

The washing steps may be done using a squeeze bottle, an automatic washing machine or a multichannel pipetting device suitable for dispensing 300 µL on each well.
After the incubation periods, the washing steps must be done following the next instructions:

Throw out the content of the plate by a brisk turn over of the plate to avoid the possible mixture of the content from one well to another.
Dispense a volume of 300 µL of washing solution on each well.
Shake delicately the plate, avoiding the contamination between wells.
Turn over the plate briskly to empty the wells.
Repeat the process as many times as are indicated in the kit´s instructions.
Prior to emptying the content of the last washing step, verify that the next reagent to be added to the plate is ready to use. Do not let the plate dry longer than strictly necessary.
After emptying the contents of the last washing step, tap the plate turned over on absorbent filter paper to remove the bulk of the remaining washing solution.

PREPARATION OF REAGENTS:

Washing solution:

Dilute one part of the concentrate washing solution provided in the kit with 24 parts of distilled or deionized water. When ready, this solution remains stable at +4°C, until the expiration date of the kit.

Dilution buffer:

Dilute one part of the concentrate dilution buffer provided in the kit with 4 parts of distilled or deionized water. When ready, this solution remains stable between +2ºC and +8°C, until the expiration date of the kit.

SAMPLES PREPARATION:

Extraction of Heat-processed products or products containing Soya.

Weigh 0.25 g of a milled food sample and place it in a 10 ml propylene tube.
Add 2.5 mL of the Extraction buffer to the tube containing the sample.
Close the tube tightly and cover the cap with parafilm to avoid evaporation.
Mix thoroughly by vortexing (5-10 seconds) and place the tubes in a rack.
Incubate the tubes between 20 and 60 minutes at room temperature. During this incubation period, vortex the sample 2-3 times.
Open the tubes, add 7.5 ml 80% ethanol, thoroughly disperse the samples by vortexing 10 to 60 seconds and incubate between 20 and 60 minutes at room temperature with agitation (a rotatory shaker at 45 turns per minute is recommended).
Centrifuge the tubes for 10 min at 2000- 2500 g at room temperature
Take the supernatants with fresh Pasteur pipettes and transfer to clean 10 mL polypropylene tubes.
The solution is now ready for ELISA.

Conjugate, Controls and Standards:
They are supplied ready to use. Do not dilute. Add 100 µL of each solution directly to the well.

Control Point
Dilute 1/20 with the Dilution Buffer (i.e. 25µL + 475µL of Dilution Buffer). Shake well before use. Only prepare the necessary volume, as the remaining volume should be discarded.

Extraction of Non Heat-processed products and products non-containing Soya.

Weigh 0.25 g of a milled, food sample and place it in a 10 ml propylene tube.
Add 10 ml of 60% ethanol and incubate between 20 and 60 minutes at room temperature with agitation.
Proceed to step 7 of the *heat processed products extraction*

Extraction of samples containing tannins or polyphenols (chocolate, red wine, fruit juices…)

In a 10 mL propylene tube, weight 0.25 g of Gelatin and 0.1 g of Polyvinyl-pyrrolidone. (Polyvinylpyrrolidone Sigma PVP-360) (Fish Gelatine Sigma No. G-7041).
Weigh 0.25 g of the milled food sample and add it to the tube.
Add 2.5 mL of Extraction Buffer
Close the tubes and seal them with parafilm in order to avoid evaporation due to heat.
Mix by vortex until the sample is completely mixed.
Incubate tubes between 20 and 60 minutes at room temperature.
Add 7.5 mL of 80% Ethanol to the sample and incubate between 20 and 60 minutes at room temperature with agitation (a rotatory shaker at 45 revolutions per minute is recommended).
Centrifuge the samples 10 minutes at 2000- 2500 g at Room Temperature.
Collect the supernatant into clean 10 mL propylene tubes.
These extracts should be stored at Room Temperature

EXTRACTION OF SWAB SAMPLES

***IMPORTANT NOTE**: this procedure can only be used for a qualitative detection of gluten (absence/presence) in a surface sample.*

Swab samples should not be used for quantification, as the initial amount of sample in the swab is unknown.

Considering that using a swab, only a little amount of sample can be taken, the extraction procedure is similar to the "normal" one, but using less volume of Extraction Buffer and Ethanol (as long as the original proportion is maintained):

Once the sample has been taken, cut the tip of the swab and put it into a 10mL propylene tube.
Add 1mL of Extraction Buffer to the tube.
Close the tube tightly with the cap and seal it using Parafilm in order to avoid evaporation.
Vortex for 5-10 seconds.
Incubate between 20 and 60 minutes at room temperature.
Add 3mL of Ethanol (80%), vortex for 10-60 seconds and incubate between 20 and 60 minutes at room temperature with agitation. IMPORTANT: do not remove the swab from the tube at this step.
Remove the swab after the incubation and use the solution for the ELISA (dilutions 1:12.5 and 1:25).
Compare the OD values of both dilutions with the OD of the lower standard, in order to have an idea about the absence/presence of Gluten on the sample surface. The results should be reported considering the Limit of Detection of the assay (3ppm of Gluten).

GENERAL INSTRUCTIONS

The extracted samples may be kept 7 days at room temperature (or 1 month at 4ºC), before performing the ELISA test. While stored, close tightly the vials with parafilm in order to avoid evaporation.

The samples should be diluted as follows before adding to the ELISA plate wells:
Samples with estimated gluten contents lower than 50 ppm: make dilutions 1:25 and 1:50, using the supplied diluent.
Samples with gluten contents between 50-100 ppm: make dilutions 1:50 and 1:100
Samples with gluten contents between 100-200 ppm: make dilutions 1:100- 1:200
Samples with gluten contents between 200-300 ppm make dilutions 1:200 and 1:400
Samples with gluten contents between 300-600 ppm make dilutions of 1:400 and 1:800.
Dilution procedure:
1:25 = 960µL of diluent+40µL of sample 1:50 = 980µL of diluent+20µL of sample 1:100 = 990µL of diluent+10µL of sample 1:200 = 1,990µL of diluent+10µL of sample 1:400 = 1,995µL of diluent+5µL of sample 1:800 = 3,995 µL of diluent+5µL of sample

TEST PROCEDURE:

All reagents must be allowed to come to room temperature before use (about 30 min.).
Add 100 µL of controls and standard solutions to duplicate wells. Add 100 µL of samples (diluted as explained above), to the remaining wells. We recommend the use of two wells per diluted sample.
Seal the plate and incubate 1 h at room temperature (22-25°C).
Wash 3 times as specified in point IV (Washing steps).
Using a multi-channel pipette, add 100 µL of specific conjugate to each well seal the plate and incubate 1 h at room temperature (22-25°C).
Wash 3 times as before.
Using a multi-channel pipette, add 100 µL of TMB substrate to each well. Keep the plate for 10 min at room temperature
Add 100 µL of stop solution to each well following the substrate's order of addition. A positive reaction will change the colour from blue to yellow
Read the OD of each well at 450 nm within 5 min after the addiction of stop solution.

READING AND INTERPRETATION OF THE RESULTS:

Validation of the test
The test could be considered valid when:
⇒ The OD of the Positive control is higher than OD of the 25ng/mL point and de OD of the Negative Control is lower than the OD of the 1.56 ng/mL point.

Interpretation:

The OD of the sample will be calculated as the arithmetic mean of the duplicates OD values.
Enter on the x-axis the mean OD values obtained for the five standards and on the y-axis the corresponding ng/ml values to represent the curve.
Sample gliadin concentration (C) should be obtained by interpolation of the OD value in the curve standard.

Sample gluten content (ppm) will be calculated as follows:

ppm of gluten = C x D x 2 x 40 / 1000

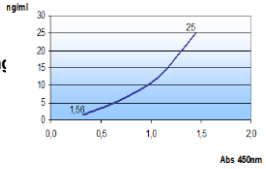
where:
C: Gliadin concentration of the sample calculated from the calibration curve in ng/mL.
D: Dilution factor of the sample (25,50,100,etc)
2: Factor applied to express the results in gluten concentration
40: Dilution factor applied in the sample preparation (0.25g in 10 ml of extraction solution).
1/1000: Conversion from ng/mL to ppm

ADDITIONAL INFORMATION

In order to obtain correct values, the OD values of the samples should be included between the OD values of the standard curve. If not, the assay should be repeated using different dilutions of the samples.
Just in case the OD value of the sample is slightly higher than the OD of the 25 ng/mL point (up to 15%), the assay is valid. In this case you have to include the positive control in the curve, as it corresponds to 50ng/ml of gliadin.
Control Point: This is an internal Control Point included in the kit in order to assure that the assay is properly working. It is not necessary to use it, but it is recommended. Procedure for analyse the Control Point:
Dilute 1:20 with Dilution Buffer and add 100µL into the correspondent well.
The OD of this point should be similar to the OD of the 25ng/mL point, with a deviation of ± 0.2
Interpolating this OD value into the standard curve (for the determination of the content of gluten), the final value should be 40±8 ppm of Gluten.

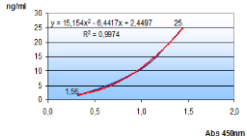
EXAMPLE:

OD of Positive Control = 1.82
OD of Negative Control = 0.13
OD of the standards points (concentration is expressed in ng
25 ng/mL = 1.44
12.5 ng/mL = 1.08
6.25 ng/mL = 0.73
3.12 ng/mL = 0.47
1.56 ng/mL = 0.32



Sample gliadin concentration (C) is obtained interpolating the sample OD value in the standard curve or using the appropriate software. For example:

Software: Excel (Microsoft)
Once obtained the gliadin standards OD values, click on "Graphic assistant"
Select type of graphic: "XY (dispersion)
Click on graphic windows and select " Add tendency line"
In label "type", click on "polynomial"
In label "Options", click on " Present formula in the graphic"
In this formula, replace the unknown factor "x" by the sample OD value.



Sample	*Dilution (D)*	*OD*	*ng/ml of gliadin (C)**	*ppm of Gluten*	*Mean (ppm of Gluten)*
A	**1:25** **1:50**	**0.31** **0.26**	**Out of limits**	**<3** **<3**	**< 3**
B	**1:50** **1:100**	**1.17** **0.85**	**15.66** **7.92**	**62.63** **63.38**	**63**
C	**1:200** **1.400**	**1.31** **0.89**	**20.02** **8.72**	**320** **279**	**299**
Control Point (32-48)	**1:20**	**1.49**	**26.49**	**42**	

*** Obtained by replacing "x" by the OD value in the tendency line.**

OVERVIEW OF PROCEDURE

Reagents must be equilibrated at room temperature (22-25ºC) before start.
Add 100µL of sample (prepared and diluted as described in point VI) in duplicated wells.
Add 100µL of standards and positive control. Cover with adhesive foil.
Incubate at room temperature (22- 25ºC) for 60 min.
Wash wells 3 times with *diluted wash* solution to remove not bound reagents.
Add 100µL of *Mab* Peroxidase Conjugate to each well. Cover with adhesive foil.
Incubate at room temperature (22- 25ºC) for 60 min.
Wash wells 3 times with *diluted wash* solution to remove un-reacted conjugate.
Add 100µL of substrate (TMB) to each well.
Incubate 10min at room temperature
Add 100µL of stop solution to each well.
Read at 450nm.

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