# VALIDATION REPORT

## Gluten Free Test



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ProGnosis BIOTECH

# Gluten Free Test Lateral Flow kit

# INDEX

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## 1. Introduction

Gluten consists of two groups of proteins, namely the glutenins and the prolamins. Prolamins are present in wheat (gliadin), barley (hordein) and rye (secalin), and are the alcohol-soluble fraction of gluten. Consumption of gluten might be harmful for people with gluten intolerance (celiac disease) and wheat allergy, because the small intestine is unable to absorb nutrients . It might cause a broad range of symptoms, such as nausea, vomiting , abdominal pain, joint pain, infertility or frequent miscarriages etc. Moreover, long-term gluten consumption may lead to more severe symptoms. For example, weakening of the bones (osteoporosis) and iron deficiency anemia.

### 1.1 Regulations

According to the Codex Alimentarius (CODEX STAN 118/1979) food labeling is divided into to groups, “gluten-free” and “very low gluten” products where the gluten content is lower than 20 mg/kg and 100 mg/kg, respectively. For more information on the allergens’ regulations, visit our website: **www.prognosis-biotech.com.**

### 1.2 Principle of the method

The presence of gluten in a sample is determined by the immunological detection of gliadin and other prolamins. Antibodies specific to gliadin are coated on the test line region (Test line) of the nitrocellulose membrane. During testing, antigens in the specimen react with the antibodies that are coated onto gold nanoparticles. The mixture migrates up the membrane to react with the antibodies immobilized on the membrane and generate a colored line in the test region T. The presence of the colored Test line indicates a positive result. In case of samples with a very high allergen concentration, the Test line fades or may not appear, giving us reduced or false negative result (hook effect phenomenon). For this purpose, a second line has been created (Hook line), whose intensity decreases as the amount of antigen increases and at very high concentrations it disappears either together with the Test line or before it. To serve as a procedural control, a colored line will always appear in the control region (Control line) if the test has been performed properly.

### 1.3. Kit Characteristics

See manual E1910/E1930 VN5

### 1.4 Method Protocol

*Sample preparation*

***Solid Samples***

- The sample must be collected according to established sampling techniques. Grind a representative sample (at least 5 g) to the particle size of fine instant coffee (50% passes through a 20 mesh screen).
- Weigh out a 1.0 g ground portion of the sample, add it into the prefilled sample tube and vortex it for 1 min. **The ratio of sample to extraction solvent is 1:5 (w/v).**
- Two (2) ml of the extract should be centrifuged at high speed for 10 min in reaction caps by using a microcentrifuge. Alternatively, let the sample settle down.
- Using a disposable pipette, transfer 1 drop from the supernatant to the empty extraction tube with the dropper tip.
- Dilute the sample **1:5** by adding 5 drops of the Matrix diluent (blue dropper tip) to the extraction tube with your supernatant.
- Close the extraction tube and shake well for a few seconds.

***Liquid Samples***

- Use 1.0 mL of the sample, add it into the prefilled sample tube and vortex it for 1 min. Follow the rest of the procedure exactly as described above for the solid samples. (You can skip using the microcentrifuge, unless your sample is a viscous liquid.)

***Surfaces and swab sampling***

- Mark out a swabbing area of approximately 10 x 10 cm.
- Moisten a swab by dipping into the extraction tube.
- Gather the sample with the swab by using a crosshatch technique. Move the swab horizontally, vertically, diagonally while rotating the tip. Repeat this starting from a different angle each time.
- After the sample collection, place the swab in the extraction tube, rotate the swab forcefully against the side of the tube for 1min. Best results are obtained when the sample is vigorously extracted in the solution. Remove the swab, squeezing the sides of the tube to extract as much liquid as possible. Shake vigorously for 1min on a vortex.
- Close the extraction tube. Add 1 drop into an empty extraction tube with the dropper tip. Dilute the sample **1:5** by adding 5 drops of the Matrix diluent (blue dropper tip).
- Close the extraction tube and shake well for a few seconds.

**NOTE 1:** The extracted sample should have a pH value of 6.2 - 7.5. If the pH is less than 6.2 as for example happens on the silage samples, the pH should be neutralized using NaOH or HCl.

**NOTE 2:** In case of cloudy, thick samples, that do not allow the mixture to develop, an extra dilution 1:1 with the Extraction Buffer is required before transferring 1 drop from the supernatant. In this case, multiply the final gluten ppm result x 2.

*iii. Immunoassay Procedure and Interpretation of results (Total time: 5min)*

- Add 3 drops in the circular window of the reaction device and allow the test to develop for 5 minutes.

***Qualitative assessment***

After 5 minutes, the test device can be visually read and interpreted as **1)Negative**, **2)Positive, 3)High Positive** or **4) Invalid**, according to a Visual result interpretation index

***Quantitative assessment***

After 5 minutes, place the reaction device inside the plastic holder in order to be scanned. Use the appropriate software to quantify results as soon as possible and no later than 1 minute after the end of analysis. The software will use a Lot specific curve to calculate the results. Refer to the Reader's manual for a detailed description of the quantification procedure.

## 2 Immunoassay Specifications

### 2.1 General Specifications

- Visual LOD of the method is 3.0 ppm (3.0mg/kg) gluten or 1.5 ppm gliadin in food samples and CIP solutions.
- Visual LOD of the method is 1µg/100 $cm^2$ on working surfaces.

### 2.2 Assay Claims

- Samples showing negative results may contain Gluten below the limit of detection of the assay. This Lateral Flow kit does not claim that food is safe for consumption based upon a determination of gluten content. Matrix effects may also affect the result of the method.
- The recovery/cross reactivity of the method might be affected when analyzing processed food (e.g. heat treatment, dehydration, etc.), because proteins may be altered or fragmented.
- Food samples that have been heat treated may contain denatured proteins which may not be captured by the antibody. Recovery of these matrices might be reduced.
- A representative sample was used for the cross reactivity evaluation. Other samples may show a different result.
- The protein content and the protein composition may differ among various species of the same matrix. Therefore, different varieties may produce different results.
- LOD in CIP solutions refers to the final rinse water. The presence of cleaning agents and detergents may affect the result of the method.

## 3. Validation

### 3.1 Determination of the Limit of Detection LOD

The lowest detectable concentration of an analyte in a method is known as LOD. In this case, we check the concentration of a freshly prepared Gliadin from Wheat Extract , extracted and measured by **Allergen-Shield Gluten S** ELISA kit,A1048/A1096. The LOD is the level at which 95% of the replicates are characterized as positive. The results of 20 replicates of 6 dilutions with Gliadin Extract are shown at the table below.

**Table 1. LOD of Gliadin proteins in liquid extract**

| Concentration (ppm) | Positive Replicates | Visual Interpretation of results |
|---|---|---|
| 100 | 20/20 | Strong Positive |
| 50 | 20/20 | Strong positive |
| 25 | 20/20 | Positive |
| 10 | 20/20 | Positive |
| 5 | 20/20 | Positive |
| 3 | 20/20 | Positive |
| 1.5 | 3/20 | Negative |

### 3.2 Specificity & Cross-Reactivity

For the determination of cross-reactivity 10 replicates of gluten-free foods were tested. The samples were analyzed according to the experimental procedure described in Chapter 1.4. Four foods containing gluten also were tested (Table 3).

**Table 2. Cross-reactivities**

| No | Commodities | % Cross Reaction (N=10) | No | Commodities | % Cross Reaction (N=10) |
|---|---|---|---|---|---|
| 1 | Almond | <LOD | 39 | Peppercorn | <LOD |
| 2 | Almond Flour | <LOD | 40 | Pistachio | <LOD |
| 3 | Amaranth Flour | <LOD | 41 | Potato Flour/ Starch | <LOD |
| 4 | Arrowroot | <LOD | 42 | Pumpkin Seed | <LOD |
| 5 | Barley | 41 | 43 | Quinoa Flour | <LOD |
| 6 | Black Bean | <LOD | 44 | Raw Shrimp | <LOD |
| 7 | Black Bean Flour | <LOD | 45 | Rice | <LOD |
| 8 | Brown Rice | <LOD | 46 | Romano Bean Flour | <LOD |
| 9 | Brown Rice Flour | <LOD | 47 | Rye | 85 |
| 10 | Buckwheat | <LOD | 48 | Sesame Seed | <LOD |
| 11 | Buckwheat Flour | <LOD | 49 | Sesame Flour | <LOD |
| 12 | Cashew | <LOD | 50 | Sorghum Flour | <LOD |
| 13 | Chestnut | <LOD | 51 | Soy Bean | <LOD |
| 14 | Chestnut Flour | <LOD | 52 | Soya Flour | <LOD |
| 15 | Chick Pea | <LOD | 53 | Spices | <LOD |
| 16 | Cocoa | <LOD | 54 | Sweet rice Flour | <LOD |
| 17 | Coconut | <LOD | 55 | Sunflower Seed | <LOD |
| 18 | Coconut Flour | <LOD | 56 | Tapioca | <LOD |
| 19 | Coffee | <LOD | 57 | Tapioca Flour/ starch | <LOD |
| 20 | Corn | <LOD | 58 | Tea | <LOD |
| 21 | Corn/Starch meal | <LOD | 59 | Triticale | 88 |
| 22 | Dried Fruits | <LOD | 60 | Walnut | <LOD |
| 23 | Egg Powder | <LOD | 61 | Wheat | 100 |
| 24 | Fava bean Flour | <LOD | 62 | Whey | <LOD |
| 25 | Flax seed Flour/ meal | <LOD | 63 | White bean Flour | <LOD |
| 26 | Garfava Flour | <LOD | 64 | White rice Flour | <LOD |
| 27 | Green Pea Flour | <LOD | 65 | Wine (merlot) | <LOD |
| 28 | Ground Beef | <LOD | 66 | Xanthan gum | <LOD |
| 29 | Ground Chicken | <LOD | 67 | Yeast (active dry) | <LOD |
| 30 | Ground Pork | <LOD | 68 | Yellow pea Flour | <LOD |
| 31 | Ground Turkey | <LOD | 69 | Lentils | <LOD |
| 32 | Guar gum | <LOD | 70 | Lentil Flour | <LOD |
| 33 | Hazelnut | <LOD | 71 | Lima Bean | <LOD |
| 34 | Hazelnut Flour | <LOD | 72 | Lima Bean Flour | <LOD |
| 35 | Kamut | >75 | 73 | Milk powder | <LOD |
| 36 | Oat Flour | <LOD | 74 | Millet | <LOD |
| 37 | Peanut | <LOD | 75 | Millet Flour | <LOD |
| 38 | Pecan | <LOD | 76 | Oats | <LOD |
| | | | | | |

All of the gluten-free matrices have no cross-reactivity at 100% with ProGnosis Biotech Gluten Free test.

Cross reactivities of the used antibodies have been determined for the pure food (e.g. corn flour). In a composed / processed food (e.g. maize bread) cross reactivities might be different.

### 3.3 Matrix Study

***Spike Protocol***

In this study we want to check the capability of our product to detect gliadin in different matrices and to confirm that the LOD of our method is 3ppm, as we described earlier in 3.1. All the samples were spiked according to AOAC INTERNATIONAL Research Institute spiking protocol. More particularly, all spike experiments were carried out by spiking the individual pre-weighed test portion with a concentration adjusted solution to maintain the spiking volume at 100 µL. The spike solution was prepared from Gliadin from wheat in ProGnosis Biotech Allergen-Shield Gluten S extraction buffer. Gliadin was weighed on an analytical balance with 0.001 g precision and dissolved in extraction buffer using a 50 mL volumetric flask. Gluten-free samples were spiked using the above prepared stock solution with a positive displacement Hamilton syringe and were left open in a fume hood for approximately 30 minutes to allow the solvent to evaporate prior extraction. For the surfaces, we chose plastic, stainless steel and synthetic (working bench) as the material. We cleaned a representative piece and we marked out 10 swabbing areas of approximately 10 x 10 cm. Each of these areas were spiked at 100µl with the stock solution and allowed to dry for 24 hours before sample collection. The resulting in-house spiked matrices and surfaces were extracted and analyzed according to the manual E1910/E1930 VN5 chapter 8.

*i. Gluten Free edible materials (Food)*

For the first Matrix Study, 3 representative gluten free matrices were spiked with the gliadin stock solution at 30ppm. Then, dilutions were made with Extraction buffer and, at each level, we used 10 replicates to check the efficiency of our product. Before we started, we confirmed that the samples did not contain gluten and that the spike level was right by using our ELISA kit Allergen-Shield Gluten S, A1048/A1096.

**Table 3. Matrix study of Gluten Free Test on food**

| Matrix | Dilution | Number of Reaction Device (N=10) | | | | | | | | | | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| Break-fast cereal | 30ppm | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/5 (6ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/10 (3ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/20 (1.5ppm) | - | - | - | + | - | - | - | + | - | - | 02/10 |
| Cake mix | 30ppm | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/5 (6ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/10 (3ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/20 (1.5ppm) | + | - | - | - | - | - | + | - | - | - | 02/10 |
| Cooked biscuit | 30ppm | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/5 (6ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/10 (3ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/20 (1.5ppm) | - | + | - | - | - | - | - | - | - | - | 01/10 |

*ii. Clean in Place Rinses (CIP SOLUTIONS)*

For this study, 2 representative CIP solutions and Deionized water were spiked with the gliadin stock solution at 30ppm. Then, dilutions were made with Extraction buffer and, at each level, we used 10 replicates to check the efficiency of our product. Before we started, we confirmed that the CIPs did not contain gluten and that the spike level was right by using our ELISA kit Allergen-Shield Gluten S, A1048/A1096.

**Table 4. Matrix study of Gluten Free Test on CIP solutions**

| Matrix | Dilution | Number of Reaction Device (N=10) | | | | | | | | | | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| Aciplus foam 2,5% | 30ppm | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/5 (6ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/10 (3ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/20 (1.5ppm) | + | - | - | - | - | - | - | - | - | + | 02/10 |
| Divosan VT5 1,8% | 30ppm | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/5 (6ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/10 (3ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/20 (1.5ppm) | - | - | - | - | - | - | + | - | - | - | 01/10 |
| Deionized water | 30ppm | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/5 (6ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/10 (3ppm) | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1/20 (1.5ppm) | - | - | - | - | - | + | - | - | - | - | 01/10 |

*iii. Surfaces-Swab Technique*

For the surfaces, we chose plastic, stainless steel and synthetic (working bench) as the material. We cleaned a representative piece and we marked out 10 swabbing areas of approximately 10 x 10 cm. Each of these areas were spiked at 100µl with the stock solution and allowed to dry for 24 hours before sample collection. For each surface, the following replicate test portions were prepared: 10 at a gliadin level of 5µg/100 $cm^2$, 10 at a level of 2µg/100 $cm^2$,10 at a level of 1µg/100 $cm^2$, and 10 at a level of 0,5µg/100 $cm^2$. The result of this study is that the LOD of our product, for environmental swabs, is 1µg/100 $cm^2$.

**Table 5. Matrix study of Gluten Free Test on surfaces**

| Surface | Spike level | Number of Reaction Device (N=10) | | | | | | | | | | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| Stainless steel | 5µg/100 $cm^2$ | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 2µg/100 $cm^2$ | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1µg/100 $cm^2$ | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 0,5µg/100 $cm^2$ | - | + | - | - | - | - | - | - | - | + | 02/10 |
| Plastic | 5µg/100 $cm^2$ | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 2µg/100 $cm^2$ | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1µg/100 $cm^2$ | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 0,5µg/100 $cm^2$ | - | - | + | - | - | - | + | - | + | - | 03/10 |
| Synthetic | 5µg/100 $cm^2$ | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 2µg/100 $cm^2$ | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 1µg/100 $cm^2$ | + | + | + | + | + | + | + | + | + | + | 10/10 |
| | 0,5µg/100 $cm^2$ | - | - | - | - | - | + | - | - | - | - | 01/10 |

### 3.4 Performance Evaluation with Reference Materials

To evaluate the efficiency of Gluten Free Test, we chose a wide range of reference materials that had been treated from FAPAS, an accredited proficiency testing provider. All samples were accompanied by certificates that confirm their assigned value. For each sample, 10 replicates were performed.

**Table 6. Evaluation of Gluten Free Test with samples prepared by FAPAS**

| Reference material | Assigned value (mg/kg) according to R-Biopharm Ridascreen Gliadin | Range for \|z\|≤2 | Result (N=10) | Average |
|---|---|---|---|---|
| FAPAS Cake Mix | 34,7 | 17,3 - 52,0 | Positive | 10/10 |
| FAPAS Cake Mix | 34,3 | 17,1 - 51,4 | Positive | 10/10 |
| FAPAS Cooked Bis- | 76,0 | 38,0 - 114,0 | Positive | 10/10 |
| FAPAS Infant Soya Formula T27240AQC | NOT DETECTED | - | <LOD | 10/10 |
| FAPAS Infant Soya Formula T27240BQC | 37,1 | 18,5 - 55,6 | Positive | 10/10 |
| FAPAS Infant Soya Formula T27264AQC | NOT DETECTED | - | <LOD | 10/10 |
| FAPAS Infant Soya Formula T27264BQC | 24,8 | 12,4 - 37,2 | Positive | 10/10 |
| FAPAS Oat Based Foodstuff T27228AQC | NOT DETECTED | - | <LOD | 10/10 |
| FAPAS Oat Based Foodstuff T27228BQC | 45,0 | 22,5 - 67,5 | Positive | 10/10 |
| FAPAS Oat Based Foodstuff T27252AQC | 16,6 | 8,3 - 24,9 | Positive | 10/10 |
| FAPAS Oat Based Foodstuff T27252BQC | NOT DETECTED | - | <LOD | 10/10 |

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