1. INTRODUCTION

Celiac disease, also known as gluten sensitive enteropathy is primarily a disease of the infant organism. It is caused by a hypersensitivity reaction in response to gliadin, a protein being present in many cereals. This, non IgE mediated food allergy leads to massive malabsorption disturbances and is characterized by a complete atrophy of the villi and a hyperplasia of the crypts of the upper intestine. Accordingly patients suffering from celiac disease must maintain a gluten free diet for the rest of their life. Gliadins are proteins containing high amounts of the amino acids proline and glutamine. These proteins belong to the nutritive tissue of the grain seeds of wheat, oat, barley and rye and are responsible for the baking properties of the flour. Due to the possibilities of the highly specific and sensitive serological determination of IgG and IgG antibodies against DGP the invasive procedures of biopsies can be given up. In the past several biopsies have been done with patients when celiac disease was suspected, after a period of a gluten-free diet and also after a specific gluten challenge. DGP antibodies titer has been proved to correlate very well with the morphological appearance of the mucosa of the upper intestine. It has been well documented that DGP antibodies level fall very quickly after a gluten free diet has begun and rise immediately after restoring gluten to the diet. Thus the serological test represents a reliable method to monitor patients, and in particular children and teenagers, for their adherence to the gluten-free diet.

2. INTENDED USE

Anti-Deamidated Gliadin Peptide (DPG) IgG kit is a solid phase enzyme immunometric assay (ELISA) designed for the quantitative measurement of IgG class antibodies directed against deamidated Gliadin peptides (DGP) in human serum or plasma. Anti-Deamidated Gliadin Peptide (DPG) IgG is intended for laboratory use only.

3. PRINCIPLE OF THE ASSAY

Anti-Deamidated Gliadin Peptide (DPG) IgG test is based on the binding of present antibodies in calibrators, controls or prediluted patient samples on the syntenic deamidated Gliadin peptides (DGP) coated on the inner surface of the wells. After a 30 minutes incubation the microplate is washed with wash buffer for removing non-reactive serum components. An anti-human IgG horseradish peroxidase conjugate solution recognizes IgG class antibodies bound to the immobilized antigens. After a 30 minutes incubation any excess enzyme conjugate, which is not specifically bound is washed away with wash buffer. A chromogenic substrate solution containing TMB is dispensed into the wells. After 15 minutes of incubation the color development is stopped by adding the stop solution. The solutions color changes into yellow. The amount of color is directly proportional to the concentration of IgG antibodies present in the original sample.

4. MATERIALS

4.1. Reagents supplied

- **Anti-DGP Coated Wells:** 12 breakapart 8-well snap-off strips coated with DGP; in resealable aluminium foil.
- **Stop Solution:** 1 bottle containing 15 ml sulphuric acid, 0.15 mol/l (avoid any skin contact), ready to use
- **Conjugate:** 1 bottle containing 15 ml Anti h-IgG conjugate with horseradish peroxidise (HRP), BSA 0.1%, Proclin < 0.0015%
- **TMB Substrate Solution:** 1 bottle containing 15 ml 3,3’,5,5’-tetramethylbenzidine 0.26 g/L, hydrogen peroxide 0.05%
- **Wash solution:** 1 bottle containing 50 ml (10x conc.) Phosphate buffer 0.2M, proclin < 0.0015%
- **Sample Diluent:** 1 bottle containing 100 ml Phosphate buffer 0.1M, NaN3 < 0.1%
- **Anti-DGP Standards:** 5 bottles, 1.2 ml each, ready to use
  - Standard 0: 0 AU/ml
  - Standard 1: 15 AU/ml
  - Standard 2: 30 AU/ml
  - Standard 3: 60 AU/ml
  - Standard 4: 240 AU/ml
- **Positive Control:** 1 bottle containing 1.2 ml, Phosphate buffer 0.1M, NaN3 < 0.1%, human serum, ready to use
- **Negative Control:** 1 bottle containing 1.2 ml, Phosphate buffer 0.1M, NaN3 < 0.1%, human serum, ready to use

4.2. Materials supplied
4.3. Materials and Equipment needed
- ELISA microwell plate reader, equipped for the measurement of absorbance at 450 nm
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Distilled water
- Disposable tubes
- Timer

5. STABILITY AND STORAGE
- Store all the kit reagents at 2-8°C. Do not freeze. Reagents are stable until the expiration date when stored and handled as directed.
- Unused antigen coated microwell strips should be resealed securely in the foil pouch containing desiccants and stored at 2-8°C.

6. REAGENT PREPARATION

6.1. Coated snap-off Strips
- The ready to use break apart snap-off strips are coated with DGP. Store at 2...8 °C. Open the bag only when it is at room temperature.
- Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2...8 °C; stability until expiry date. Do not remove the adhesive sheets on the unused strips.

6.2. Anti-DGP Standards / control
- Since no international reference preparation for Anti-DGP antibodies is available, the assay system is calibrated in relative arbitrary units. The standards have approximately the following concentration:

<table>
<thead>
<tr>
<th>S0</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
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</thead>
<tbody>
<tr>
<td>AU/mL</td>
<td>0</td>
<td>15</td>
<td>30</td>
<td>60</td>
</tr>
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</table>

6.3. TMB Substrate Solution
- The bottle contains 15 ml of 3,3',5,5'-tetramethylbenzidine 0,26 g/L, hydrogen peroxide 0,05%. The reagent is ready to use and has to be stored at 2...8°C in the dark. The solution should be colourless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away.

6.4. Stop Solution
- The bottle contains 15 ml 0.15 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2...8°C.

6.5. Wash Solution
- Dilute the content of each vial of the buffered wash solution concentrate (10X) with distilled water to a final volume of 500 mL prior to use. For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C.

6.6. Sample Diluent
- The bottle contains 100 ml Phosphate buffer 0,1M, NaN3 < 0,1%

6.7. Conjugate IgG
- The bottle containing 15ml Anti h-IgG conjugate with horseradish peroxidise (HRP), BSA 0,1%, Proclin < 0,0015%

7. SPECIMEN COLLECTION AND PREPARATION

For determination of Anti-DGP human serum or plasma are the preferred sample matrixes.

All serum and plasma samples have to be prediluted with sample diluent 1 : 100. Therefore 10 L of sample may be diluted with 990L of sample diluent. The patients need not to be fasting, and no special preparations are necessary. Collect blood by venipuncture into vacutainers and
separate serum (after clot formation) or plasma from the cells by centrifugation.

Samples may be stored refrigerated at 2-8 °C for at least 5 days. For longer storage of up to six months samples should be stored frozen at -20°C. To avoid repeated thawing and freezing the samples should be aliquoted. Neither Bilirubin nor Hemolysis have significant effect on the procedure. The Controls are ready to use.

8. ASSAY PROCEDURE

8.1. Test Preparation

Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder. Please allocate at least:

1 well (e.g. A1) for the substrate blank 2 wells (e.g. B1+C1) for standard 0 2 wells (e.g. D1+E1) for standard 1 2 wells (e.g. F1+G1) for standard 2 2 wells (e.g. H1+A2) for standard 3 2 wells (e.g. B2+C2) for standard 4 2 wells (e.g. D2+E2) for positive control 2 wells (e.g. F2+G2) for negative control

It is recommended to determine standards and patient samples in duplicate. Perform all assay steps in the order given and without any appreciable delays between the steps. A clean, disposable tip should be used for dispensing each standard and each patient sample.

8.2. Test Procedure

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Standard</th>
<th>Sample or Controls</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard S0-S4</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>100 µL</td>
<td></td>
</tr>
<tr>
<td>Diluted Sample</td>
<td></td>
<td>100 µL</td>
<td></td>
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</tbody>
</table>

Incubate 30 minutes at room temperature (22-28°C). Remove the contents from each well, wash the wells three times with 300 µL of diluted wash solution.

<table>
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<tbody>
<tr>
<td>Conjugate</td>
<td>100 µL</td>
<td>100 µL</td>
<td></td>
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Incubate 30 minutes at room temperature (22-28°C). Remove the contents from each well, wash the wells three times with 300 µL of diluted wash solution.

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<th>Sample or Controls</th>
<th>Blank</th>
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</thead>
<tbody>
<tr>
<td>TMB Substrate</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Incubate 15 minutes in the dark at room temperature (22-28°C).

<table>
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<th>Reagent</th>
<th>Standard</th>
<th>Sample or Controls</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stop solution</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Shake the microplate gently Read the absorbance (E) at 450 nm against Blank.

9. RESULTS

For Anti-DGP IgG a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice. Smoothed-Spline Approximation and log-log coordinates are also suitable. However, we recommend using a Lin-Log curve. First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight
line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

Typical Results (example only) The figures below show typical results for Anti-DGP IgG. These data are intended for illustration only and should not be used to calculate results from another run.

<table>
<thead>
<tr>
<th>N</th>
<th>ODI</th>
<th>OD2</th>
<th>mean</th>
<th>C1</th>
<th>C2</th>
<th>mean</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD0</td>
<td>0.011</td>
<td>0.011</td>
<td>0.011</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>6E-7</td>
</tr>
<tr>
<td>STD1</td>
<td>0.165</td>
<td>0.161</td>
<td>0.163</td>
<td>14.74</td>
<td>14.36</td>
<td>14.55</td>
<td>1.89</td>
</tr>
<tr>
<td>STD2</td>
<td>0.322</td>
<td>0.324</td>
<td>0.323</td>
<td>30.46</td>
<td>30.67</td>
<td>30.57</td>
<td>0.48</td>
</tr>
<tr>
<td>STD3</td>
<td>0.590</td>
<td>0.590</td>
<td>0.590</td>
<td>59.78</td>
<td>59.78</td>
<td>59.78</td>
<td>4E-7</td>
</tr>
<tr>
<td>STD4</td>
<td>1.694</td>
<td>1.768</td>
<td>1.731</td>
<td>232.1</td>
<td>248.1</td>
<td>240.1</td>
<td>4.71</td>
</tr>
</tbody>
</table>

10. REFERENCE VALUES
In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Anti-DGP tests:

anti-DGP-Ab IgG [AU/ml]
- Negative: < 15
- Equivocal: 15 - 30
- Positive: > 30

Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually. It is recommended that each laboratory establishes its own normal and pathological ranges of serum Anti-DGP.

11. SPECIFIC PERFORMANCE CHARACTERISTICS

11.1. Precision
Intra-Assay Within run variation was determined by replicate 16 times two different sera with values in the range of standard curve. The within assay variability is ≤ 3.8%.

Inter-Assay Between run variation was determined by replicate the measurements of two different control sera with different lots of kits and/or different mix of lots of reagents. The between assay variability is ≤ 7.8%.

11.2. Sensitivity
Comparison test against a commercial reference kit, performed on 63 sera (32 of them positive sera and 31 negative sera) showed a 94.1% sensitivity.

11.3. Specificity
Comparison test against a commercial reference kit, performed on 63 sera (32 of them positive sera and 31 negative sera) showed a 100.0% specificity.

11.4. Detection limit
The lowest concentration of anti-DGP IgG that can be distinguished from zero standard is 0.13 AU/mL with a confidence limit of 95%.

12. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

13. WARNINGS AND PRECAUTIONS
This kit is intended for research use by professional persons only. Use appropriate personal protective equipment while working with the reagents provided. All human source material used in the preparation of standards and controls for this product has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the Standard and the Controls should be handled in the same manner as potentially infectious material. Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious. Some reagents contain small amounts of Sodium Azide (NaN3) or Proclin 300R as preservatives. Avoid the contact with skin or mucosa. Sodium Azide may be toxic if ingested or absorbed through the skin or eyes; moreover it may react with lead or copper plumbing to form potentially explosive metal azides. If you use a sink to remove the reagents, allow scroun through large amounts of water to prevent azide build-up. The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes. The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes. Avoid the exposure of reagent TMB/H2O2 to directed sunlight, metals or oxidants. PRECAUTIONS Please adhere strictly to the sequence of pipetting steps.
provided in this protocol. All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use. Do not interchange kit components from different lots. The expiry dates printed on the labels of the box and of the vials must be observed. Do not use any kit component beyond its expiry date. WARNING: the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly; therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.). For divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, for doses dispensed with the aid of automatic and semi-automatic devices, before using the conjugate, it is advisable to clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommended when the kit is processed using analyzers which are not equipped with disposable tips. If you use automated equipment, it is your responsibility to make sure that the kit has been appropriately tested. The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background. It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond 10 minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate. Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction. Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. Maximum precision is required for reconstitution and dispensation of the reagents. Samples microbiologically contaminated should not be used in the assay. Highly lipemic or haemolysed specimens should similarly not be used. Plate readers measure vertically. Do not touch the bottom of the wells.

14. BIBLIOGRAPHY

### SCHEME OF THE ASSAY

**Ant-DGP**

**Test Preparation**

Prepare reagents and samples as described. Establish the distribution and identification plan for all specimens and controls on the resultsheet supplied in the kit.

Select the required number of microtiter strips or wells and insert them into the holder.

**Assay Procedure**

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