BACKGROUND

Coeliac disease is an inflammatory disease of the small intestine caused by ingestion of wheat gluten or related proteins from rye or barley. In the small intestine, ingested gluten is broken down into gliadin peptides which are deamidated by the enzyme tissue transglutaminase (Tg) resulting in the substitution of glutamine amino acid residues with glutamic acid. These modified gliadin peptides produced by this deamidation reaction are crucial to the toxic mechanism of coeliac disease and enhance the immunogenicity of gliadin, provoking an immune response. In coeliac disease, presentation of deamidated gliadin peptides to the immune system results in the production of IgA and IgG antibodies against gliadin and other auto-antigens resulting in a local intense inflammatory reaction leading to loss of villi and a dramatic reduction in the capacity to absorb nutrients.

Conventional ELISA methods for measuring antibodies to gliadin use crude gliadin (the ethanol soluble fraction of gluten). These assays show limited sensitivity and specificity for the diagnosis of coeliac disease. Recent studies have shown that gliadin reactive antibodies bind a limited number of specific epitopes on the gliadin molecule and that deamidation of gliadin enhances the binding of anti-gliadin antibodies present in the serum of coeliac disease patients. Therefore assays using specific deamidated gliadin peptides have demonstrated improved diagnostic accuracy compared to the ELISA assays using a crude gliadin antigen preparation.

The measurement of antibodies against gliadin is useful in monitoring disease activity and compliance to a gluten free diet. Anti-gliadin antibodies are also helpful in early detection of coeliac disease as these antibodies are produced early on in the disease. Gliadin IgA antibodies are thought to be more sensitive markers of coeliac disease compared with the IgG class while gliadin IgG antibodies are more specific. It is recommended that both IgA and IgG antibody classes should be measured, due to the high incidence of IgA deficiency among coeliac patients, as an IgA deficiency may serologically indicate the expected performance of the batch.

INTENDED USE

These assays are designed for the in-vitro measurement of specific IgG or IgA antibodies against a modified gliadin peptide (MGP) in human serum from patients with coeliac disease.

Sufficient materials are supplied to allow a maximum of 890 samples to be tested in single or 410 in duplicate, with a calibration curve and a positive and negative control yielding semi-quantitative results. If used as a screening assay, 930 samples in single or 450 in duplicate can be tested together with the cut-off, positive and negative control.

This kit can be run either as a semi-quantitative assay, or as a screening assay, using the cut-off control provided in place of the calibrators.

BACKGROUND

Coeliac disease is an inflammatory disease of the small intestine caused by ingestion of wheat gluten or related proteins from rye or barley. In the small intestine, ingested gluten is broken down into gliadin peptides which are deamidated by the enzyme tissue transglutaminase (Tg) resulting in the substitution of glutamine amino acid residues with glutamic acid. These modified gliadin peptides produced by this deamidation reaction are crucial to the toxic mechanism of coeliac disease and enhance the immunogenicity of gliadin, provoking an immune response. In coeliac disease, presentation of deamidated gliadin peptides to the immune system results in the production of IgA and IgG antibodies against gliadin and other auto-antigens resulting in a local intense inflammatory reaction leading to loss of villi and a dramatic reduction in the capacity to absorb nutrients.

Conventional ELISA methods for measuring antibodies to gliadin use crude gliadin (the ethanol soluble fraction of gluten). These assays show limited sensitivity and specificity for the diagnosis of coeliac disease. Recent studies have shown that gliadin reactive antibodies bind a limited number of specific epitopes on the gliadin molecule and that deamidation of gliadin enhances the binding of anti-gliadin antibodies present in the serum of coeliac disease patients. Therefore assays using specific deamidated gliadin peptides have demonstrated improved diagnostic accuracy compared to the ELISA assays using a crude gliadin antigen preparation.

The measurement of antibodies against gliadin is useful in monitoring disease activity and compliance to a gluten free diet. Anti-gliadin antibodies are also helpful in early detection of coeliac disease as these antibodies are produced early on in the disease. Gliadin IgA antibodies are thought to be more sensitive markers of coeliac disease compared with the IgG class while gliadin IgG antibodies are more specific. It is recommended that both IgA and IgG antibody classes should be measured, due to the high incidence of IgA deficiency among coeliac patients, as an IgA deficiency may serologically mask the disease.

3  PRINCIPLE OF THE ASSAY

Microwells are pre-coated with a modified gliadin peptide. Calibrators, controls and diluted patient samples are added to the wells and antibodies recognising the modified gliadin peptide bind during the first incubation. After washing the wells to remove all unbound proteins, purified peroxidase labelled rabbit anti-human IgG or IgA (γ or α chain specific) conjugate is added. The conjugate binds to the captured human antibody and the excess unbound conjugate is removed by a further wash step. The bound conjugate is visualised with 3,3',5,5' tetramethylbenzidine (TMB) substrate which gives a blue reaction product, the intensity of which is proportional to the concentration of antibody in the sample. Phosphoric acid is added to each well to stop the reaction. This produces a yellow end point colour, which is read at 450nm.

4  PRECAUTIONS

4.1 WARNING

- All human sera supplied have been tested at donor level and found negative for Hepatitis B surface antigens and antibodies to HIV 1 and 2 and Hepatitis C virus. However, these tests cannot guarantee the absence of infectious agents. Proper handling and disposal methods should be established and only personnel qualified in handling of potentially infectious materials should be permitted to use this kit.
- Sodium azide may react with lead and copper plumbing to form explosive metal azides. On disposal of reagents flush with a large volume of water, to prevent azide build-up.
- The buffers and serum supplied in this kit contain various enzyme inhibitors as listed below. These should be handled with care.

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kathon</td>
<td>0.02%</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>0.099%</td>
</tr>
<tr>
<td>ProClin™ 300</td>
<td>0.045%</td>
</tr>
<tr>
<td>Bromonitrooxide</td>
<td>0.002%</td>
</tr>
<tr>
<td>Methylsulphoxide</td>
<td>0.002%</td>
</tr>
</tbody>
</table>

ProClin™ is a trademark of Rohm and Haas Corp. Philadelphia, PA.

- Kathon is an irritant and may cause sensitisation by skin contact.
- The stop solution contains 3M phosphoric acid, which is an irritant. Avoid contact with skin and eyes.
- Reagent spills should be cleaned up appropriately, observing local and environmental regulations.

4.2 CAUTION

- These products should only be used by appropriately trained personnel.
- Strict adherence to the protocol is recommended. Any deviation may affect assay performance, and the results obtained. Pay attention to specific Notes and warnings throughout these Instructions for Use.
- Calibrator, control, conjugate and plate batch numbers are not interchangeable. Substitution of such components with batch numbers that differ from those that are provided in the kit could lead to inconsistent and inaccurate results. All strips used must be taken from the same foil pack.
- To avoid reagent contamination, only use new or clean plastic / glassware. Never return unused reagents to the bottles.
- Do not leave reagent bottles uncapped; any resulting evaporation or contamination will lead to inconsistent results.
- TMBS substrate must not be exposed to light or water.
- Microbiologically contaminated, haemolysed or lipaemic serum and specimens containing particulate matter should not be used.
- Inaccurate sample dilution cannot be checked, as kit controls are ready to use. The use of calibrated pipettes and appropriate internal QC samples is recommended.
- The use of automated assay systems, sample dilutors and other automated equipment may lead to differences in results when compared to the manual procedure. It is the responsibility of any laboratory to fully validate the system, and ensure the results fall within the limits as defined in this insert and associated QC certificate.
- All equipment used must be calibrated and maintained according to the manufacturer’s instruction.

4.3 STORAGE AND STABILITY

- The kit should be stored at 2-8°C and should not be frozen. Inappropriate storage temperatures will affect the results.
- Diluted wash buffer can be stored at 2-8°C for a maximum of four weeks, and can be used direct from cold without affecting assay performance.
- The expiry date of the kit is shown on the outer label.

5  SAMPLE COLLECTION AND STORAGE

- Blood samples should be collected by venepuncture allowed to clot naturally and the serum separated.
- The serum may be stored at 2-8°C for up to 7 days prior to assay, or for prolonged storage, aliquoted and stored at -20°C or below.
- Repeated thawing and freezing should be avoided.
- Serum samples should not be heat-inactivated, as this may give false positive results.

6  MATERIALS

6.1 MATERIALS SUPPLIED - Common to all kits

- Instruction Leaflet: Giving full assay details.
- QC Certificate: Indicating the expected performance of the batch.
- Gliadin (MGP) Coated Wells: 10 plates each containing 12 breakapart 8 well strips coated with a modified gliadin peptide. Each plate is packaged in a re-sealable foil bag containing two desiccant pouches.
- Type II Sample Diluent: 19 bottles containing 50mL of buffer for sample dilution. Coloured yellow, ready to use.
- Type III Wash Buffer: 3 bottles containing 50mL of a 20-fold concentrated buffer for washing the wells.
- TMB Substrate: 10 bottles containing 14mL TMB substrate. Ready to use.

Stop Solution: 10 bottles containing 14mL of 3M phosphoric acid. Ready to use.
6.2 PRODUCT SPECIFIC MATERIALS SUPPLIED

6.2.1 BINDAZYM/E Anti-gliadin (MGP) IgG (MK136.10.U)
- **Gliadin (MGP) IgG Calibration:** 5 sets of 5 bottles each containing 1mL of diluted human serum, with the following concentrations of anti-gliadin (MGP) IgG antibody: 100, 33.3, 11.1, 3.7, 1.23 U/mL. Ready to use.
- **Gliadin (MGP) IgG Cut-off Control:** 5 bottles containing 1mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
- **Gliadin (MGP) IgG Positive Control:** 5 bottles containing 1mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
- **Gliadin (MGP) IgG Negative Control:** 5 bottles containing 1mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
- **Gliadin (MGP) IgG Conjugate:** 10 bottles containing 12mL of purified peroxidase labelled antibody to human IgG. Coloured red, ready to use.

6.2.2 BINDAZYM Anti-gliadin (MGP) IgA (MK136.10.U)
- **Gliadin (MGP) IgA Calibrators:** 5 sets of 5 bottles each containing 1mL of diluted human serum, with the following concentrations of anti-gliadin (MGP) IgA antibody: 100, 33.3, 11.1, 3.7, 1.23 U/mL. Ready to use.
- **Gliadin (MGP) IgA Cut-off Control:** 5 bottles containing 1mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
- **Gliadin (MGP) IgA Positive Control:** 5 bottles containing 1mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
- **Gliadin (MGP) IgA Negative Control:** 5 bottles containing 1mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
- **Gliadin (MGP) IgA Conjugate:** 10 bottles containing 12mL of purified peroxidase labelled antibody to human IgA. Coloured green, ready to use.

6.3 ADDITIONAL MATERIALS AND EQUIPMENT – not supplied
- Automatic microplate plate washer: This is recommended, however, plate washing can be performed manually.
- Plate reader: Capable of measuring optical densities at 450nm referenced on air.
- Distilled or deionised water: This should be of the highest quality available.
- Calibrated micropipettes: For dispensing 100µL volumes of conjugate, substrate and stop solution.
- Glass/plastic tubes: For sample dilution.

7 ASSAY METHOD

7.1 PRE-ASSAY STEPS
1. Bring the kit to room temperature
   - The kit is designed for room temperature operation (20-24°C).
   - Remove the kit from storage and stand at room temperature for approximately 60 minutes. Wells must not be removed from the foil bag until they have reached room temperature.
2. Component mix
   - Gently mix each kit component before use.
3. Wash buffer dilution
   - Add 50mL of the wash buffer concentrate to 950mL of distilled water (1 in 20 dilution) into a clean container and mix. Smaller volumes can be diluted as appropriate.
   - Diluted wash buffer can be stored at 2-8°C for up to 4 weeks, therefore only dilute the appropriate amount. If the buffer shows any sign of microbial contamination or turns cloudy, discard and prepare a fresh solution.
4. Sample dilution
   - Dilute 10µL of each sample with 1000µL of sample diluent (1:100) and mix well.
   - Diluted sample must be used within 8 hours.
5. Strip and frame handling
   - Place the required number of wells in the strip holder. Position from well A1, filling columns from left to right across the plate. When handling the plate, squeeze the long edges of the frame to prevent the wells falling out.
   - Return unused wells to the foil bag immediately with the two desiccant pouches and re-seal tightly to minimise exposure to moisture. Take care not to puncture or tear the foil bag, see below.
   - WARNING: Exposure of wells to moisture or contamination by dust or other particulate matter will result in antigen degradation, leading to poor assay precision and potentially false results.

7.2 ASSAY METHOD
1. Sample addition
   - Dispense 100µL of each calibrator (semi-quantitative assay) or cut-off control (screening assay), assay controls and diluted (1:100) sample into the appropriate wells of the plate provided.
   - Note: Samples should be added as quickly as possible to the plate to minimise assay drift, and the timer started after the addition of the last sample.
   - Incubate at room temperature for 30 minutes.
2. Washing
   - The washing procedure is critical and requires special attention. An improperly washed plate will give inaccurate results, with poor precision and high backgrounds.
   - After incubation remove the plate and wash wells 3 times with 250-350µL wash buffer per well. Wash the plate either by using an automatic plate washer or manually as indicated below. After the final automated wash, invert the plate and tap the wells dry on absorbent paper.
   - Plates can be washed manually as follows:
     a. Gently shake the plate on a flat surface.
     b. Repeat step a twice.
     c. Repeat a and b.
3. Substrate (TMB) addition
   - Dispense 100µL of TMB substrate into each well, blot the top of the wells with a tissue to remove any splashes.
   - Note: To avoid contamination never return excess TMB to the reagent bottle.
   - Incubate at room temperature in the dark for 30 minutes.
4. Stopping
   - Dispense 100µL of stop solution into each well. This causes a change in colour from blue to yellow.
5. Optical density measurement
   - Read the optical density (OD) of each well at 450nm on a microplate reader, within 30 minutes of stopping the reaction.

8 RESULTS AND QUALITY CONTROL

8.1 SEMI-QUANTITATIVE / SCREENING ASSAYS
1. Quality assessment of the optical densities
   - In order for an assay to be valid, all the following criteria must be met:
     a. Calibrators / cut-off control (as applicable), positive and negative controls must be included in each run.
     b. The values obtained for the positive and negative controls should be in the ranges specified on the QC Certificate.
     c. The curve shape should be similar to the calibration curve, shown on the QC Certificate.
   - The above criteria are not met, the assay is invalid and the test should be repeated.
2. Calculate the mean optical densities (For assays run in duplicate only)
   - For each calibrator, control and sample calculate the mean OD of the duplicate readings. The percentage coefficient of variation (% CV) for each duplicate OD should be less than 15%.

8.2 SCREENING ASSAY – CALCULATION OF RESULTS
1. Quality assessment of the optical densities
   - Calculate OD values for each calibrator / cut-off control (as applicable), positive and negative controls
   - Plot the OD values against the OD on the linear scale for each calibrator:
     - Automatic – use appropriately validated software, and the curve fit that best fits the data.
     - Manual – using log/linear graph paper, draw a smooth curve through the points (not a straight line or point to point).

2. Results interpretation
   - Using the cut-off control, interpret the results according to the following table:

<table>
<thead>
<tr>
<th>RESULT</th>
<th>INTERPRETATION</th>
<th>ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD equal to or greater than cut-off control</td>
<td>Suspected of having anti-gliadin (MGP) antibodies</td>
<td>Test in the semi-quantitative assay</td>
</tr>
<tr>
<td>OD less than cut-off control</td>
<td>Negative</td>
<td>Report as negative</td>
</tr>
</tbody>
</table>

8.3 SEMI-QUANTITATIVE ASSAY – CALCULATION OF RESULTS
1. Plot calibration curve
   - The calibration curve can be plotted either automatically or manually as follows by plotting the anti-gliadin (MGP) antibody concentration on the log scale against the OD on the linear scale for each calibrator:
     a. Automatic – use appropriately validated software, and the curve fit that best fits the data.
     b. Manual – using log/linear graph paper, draw a smooth curve through the points (not a straight line or point to point).

2. Treatment of anomalous points
   - If any one point does not lie on the curve, it can be removed. If the absence of this point means that the curve has a shape dissimilar to that of the sample calibration curve, or more than one point appears to be anomalous, then the assay should be repeated.
3. Calculation of the control values
Read the level of the anti-gliadin (MGP) antibody from the calibration curve. The values should fall within the ranges given on the QC Certificate.

4. Calculation of antibody levels in diluted samples
Read the level of the anti-gliadin (MGP) antibody in the diluted samples directly from the calibration curve.

Note: The calibrator values have been adjusted by a factor of 100 to account for a 1:100 sample dilution, therefore no further conversion is required.

5. Assay calibration
The assay is calibrated in U/mL against an arbitrary reference calibrator, as no internationally recognised reference preparation is currently available.

6. Limitations
• The results obtained from this assay are not diagnostic proof of the presence or absence of disease.
• A negative anti-gliadin IgA result cannot be used to exclude coeliac disease, especially if accompanied with a positive IgG anti-gliadin, since the incidence of overall IgA deficiency is raised in these patients.
• Anti-gliadin antibodies are not exclusive to coeliac disease, having been detected in patients with ulcerative colitis and those with allergy to cereals.
• The use of this assay with paediatric samples has not been established.

9. EXPECTED VALUES
The results obtained from this assay are not diagnostic proof of the presence or absence of disease.

Anti-gliadin IgA

| Sample 1 | 6.2 |
| Sample 2 | 10.7 |
| Sample 3 | 15.2 |
| Sample 4 | 21.3 |
| Sample 5 | 33.3 |
| Sample 6 | 54.5 |

10 PERFORMANCE CHARACTERISTICS

10.1 PRECISION

INTRA-ASSAY PRECISION
The intra-assay precision was measured using six samples tested 20 times each. The % CV for each sample is given below:

<table>
<thead>
<tr>
<th>Anti-gliadin IgG</th>
<th>n=6</th>
<th>Concentration (U/mL)</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td></td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Sample 2</td>
<td></td>
<td>10.6</td>
<td>8.8</td>
</tr>
<tr>
<td>Sample 3</td>
<td></td>
<td>14.2</td>
<td>5.3</td>
</tr>
</tbody>
</table>

INTER-ASSAY PRECISION
The inter-assay precision was measured using six samples tested in duplicate six times over three/four days. The %C.V. for each sample is given below:

<table>
<thead>
<tr>
<th>Anti-gliadin IgG</th>
<th>n=6</th>
<th>Concentration (U/mL)</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td></td>
<td>6.9</td>
<td>4.1</td>
</tr>
<tr>
<td>Sample 2</td>
<td></td>
<td>10.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Sample 3</td>
<td></td>
<td>13.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Sample 4</td>
<td></td>
<td>22.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Sample 5</td>
<td></td>
<td>42.3</td>
<td>4.0</td>
</tr>
<tr>
<td>Sample 6</td>
<td></td>
<td>69.7</td>
<td>6.5</td>
</tr>
</tbody>
</table>

10.2 ANALYTICAL SENSITIVITY
Assay sensitivity of 1.23 U/mL was confirmed by assaying two samples in multiple replicates with values of 1.2 and 1.8 (Gliadin IgG) and 1.4 and 1.8 (Gliadin IgA) times the lowest calibrator point (1.23 U/mL). Statistical analysis by the Student’s t test confirmed that these samples were significantly different from each other (p<0.0001).

10.3 MEASURING RANGE
The measuring range of the assays is 1.23-100 U/mL.

10.4 NORMAL RANGE
Anti-gliadin IgA and IgG autoantibody levels were measured in serum from 200 normal adult blood donors. Six were positive for gliadin IgA and three for gliadin IgG autoantibodies (Section 10.4). The same 200 samples tested in another 510k approved gliadin peptide assay yielded 14 positives for IgA gliadin antibodies, of which 4 were common to both kits. For IgG, just one positive was obtained and this was in agreement with the BINDAZYME result, whilst a second was close to the border in both kits. All the positive normal samples detected by the 4 kits were negative for both ITG IgG and ITG IgA antibodies.

The ranges are provided as a guide only. ELISA assays are very sensitive and capable of detecting small differences in sample populations. It is recommended that each laboratory determine its own normal range, based on the population techniques and equipment employed.

10.5 RELATIVE SPECIFICITY, SENSITIVITY, AGREEMENT
The relative specificity, sensitivity and agreement has been determined against alternative anti-gliadin IgG and IgA EIA kits using test samples respectively. The relative specificity, sensitivity and agreement have been determined against alternative antigliadin IgG and IgA EIA kits using test samples from the following patients: biopsy-confirmed coeliac (100), diabetes (21), rheumatoid arthritis (16) or with the following auto-antibodies: actin (4), AMR (6), centromere (5), LKM (4), histone (4), SSB (4), Sm-RNP (4), GBM (4), B2GP1 IgG (2), IgA (1), IgM (1), fibrillarin (1), chromatin (1), and PCNA (1).

All but one of the BINDAZYME positive samples were in the coeliac group (positive incidence of 53%).

6/7 of the discrepant samples were in the coeliac group, and 6/7 tested positive in the BINDAZYME tissue Transglutaminase (TG) IgG and/or IgA assays.

Fifty-three BINDAZYME positive results were obtained in the coeliac group, giving a positive incidence of 53% in coeliac patients. All of the 4 remaining BINDAZYME positive samples were amongst the 7 positives detected in the non-coeliac group by the alternative IgA gliadin peptide assay. *All 3 samples were positive for ITG IgA, whilst * 6/10 of the other discrepant samples were negative for ITG IgA antibodies.
10.6 INTERFERING SUBSTANCES

A range of interfering substances were spiked into anti-gliadin IgA and IgG negative and positive samples, which were subsequently tested on the gliadin IgA and IgG assays. The method used to check these substances was based on the Interference Check A Plus™, Kokusai, Japan.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin F (Free)</td>
<td>19.1mg/dL</td>
</tr>
<tr>
<td>Bilirubin C (Conjugate)</td>
<td>21.6mg/dL</td>
</tr>
<tr>
<td>Haemolysed Haemoglobin</td>
<td>494mg/dL</td>
</tr>
<tr>
<td>Chyle</td>
<td>1590 Units</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>50 IU/mL</td>
</tr>
</tbody>
</table>

No interference by these substances was observed in any of the samples tested.

10 REFERENCES


Plate Template

Please see back of insert.