Intended Use
This assay is designed for the in-vitro measurement of specific IgA autoantibodies against tissue transglutaminase (tTG) present in human serum, as an aid in the diagnosis of Coeliac disease. Sufficient materials are supplied to allow a maximum of 41 samples to be tested in duplicate or 89 in single, with a calibration curve and 2 controls.

Summary and Explanation of the test
Coeliac disease is characterised by gluten intolerance leading to a chronic malabsorptive disorder due to inflammation of the intestinal mucosa and flattening of the epithelium. The revised criteria for the diagnosis of coeliac disease, according to The European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) 1990, requires the identification of a single positive gut biopsy together with the demonstration of at least two of the three IgA autoantibodies detailed below. Autoantibodies associated with coeliac disease include; IgA anti-endomysial antibodies, IgG and IgA anti-gliadin antibodies and anti-reticulin antibodies. Several studies have demonstrated that IgA anti-endomysial tests have a greater than 99% specificity for coeliac disease with a greater sensitivity than anti-gliadin and anti-reticulin assays. The target endomysial antigen has recently been identified as the calcium dependent, protein cross-linking, enzyme tissue transglutaminase. The ELISA assay for IgA anti-tTG provides a suitable alternative to immunofluorescent assays (IFA) and is ideal for small and large-scale screening. The use of recombinant human tissue transglutaminase as a source of antigen is reviewed in references 11 and 12.

Principles of the Procedure
Microwells are pre-coated with recombinant human tTG antigen, the antigen has been expressed in Baculovirus cells and the expression construct used a cDNA coding for the long spliced isoform of human tTG. The calibrators, controls and diluted patient samples are added to the wells and autoantibodies recognising the tTG antigen bind during the first incubation. After washing the wells to remove all unbound proteins, purified peroxidase labelled goat anti-human IgA conjugate is added. The conjugate binds to the captured human autoantibody and the excess unbound conjugate is removed by a further wash step. 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 autoantibody in the sample. Sulfuric acid is added to each well to stop the reaction. This produces a yellow end point colour, which is read at 450nm.

Reagents
1. Polystyrene microwell ELISA plate coated with recombinant tTG antigen (12-1 x 8 wells), with holder in foil package containing desiccants
2. ELISA Negative Control, 1 vial of buffer containing preservative and human serum with no IgA human antibodies to tTG, prediluted, 1.2mL
3. R h-tTG IgA ELISA Calibrator A, 1 vial of buffer containing preservative and human IgA serum antibodies to tTG, prediluted, 1.2mL
4. R h-tTG IgA ELISA Calibrator B, 1 vial of buffer containing preservative and human IgA serum antibodies to tTG, prediluted, 1.2mL
5. R h-tTG IgA ELISA Calibrator C, 1 vial of buffer containing preservative and human IgA serum antibodies to tTG, prediluted, 1.2mL
6. R h-tTG IgA ELISA Calibrator D, 1 vial of buffer containing preservative and human IgA serum antibodies to tTG, prediluted, 1.2mL
7. R h-tTG IgA ELISA Calibrator E, 1 vial of buffer containing preservative and human IgA serum antibodies to tTG, prediluted, 1.2mL
8. R h-tTG IgA Positive Control, 1 vial of buffer containing preservative and human IgA serum antibodies to tTG, prediluted, 1.2mL
9. HRP Sample Diluent, 2 vials – colored pink containing Tris-buffered saline, Tween 20, protein stabilizers and preservative, 50mL
10. HRP Wash Concentrate, 1 vial of 40x concentrate - colored red containing Tris-buffered saline and Tween 20, 25mL. Refer to the Methods Section for dilution instructions.
11. HRP R h-tTG IgA Conjugate, anti-human IgA, 1 vial – colored yellow containing buffer, protein stabilizers and preservative, 10mL
12. TMB Chromogen, 1 vial containing stabilizers, 10mL
13. HRP Stop Solution, 0.344M Sulfuric Acid, 1 vial – colorless, 10mL

Warnings
1. WARNING: This product contains a chemical (0.02% chloramphenicol) in the sample diluent, controls, and conjugate known to the State of California to cause cancer.
2. All human source material used in the preparation of controls for this product has been tested and found negative for antibody to HIV, HBsAg, and HCV by FDA cleared methods. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the R h-tTG IgA Control, R h-tTG IgA Calibrators A through E and ELISA Negative Control should be handled in the same manner as potentially infectious material.
3. Sodium Azide is used as a preservative. Sodium Azide is a poison and may be toxic if ingested or absorbed through the skin or eyes. Sodium azide may react with lead or copper plumbing to form potentially explosive metal azides. Flush sinks, if used for reagent disposal, with large volumes of water to prevent azide build-up.
4. The HRP conjugate contains a dilute poisonous/corrosive chemical, which may be toxic if ingested in large amounts. To prevent possible chemical burns, avoid contact with skin and eyes.
5. TMB Chromogen 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.
6. The HRP Stop Solution consists of a dilute sulfuric acid solution. Avoid exposure to bases, metals, or other compounds, which may react with acids. Sulfuric acid is a poison and corrosive, which may be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
7. Use appropriate personal protective equipment while working with the reagents provided.
8. Spilled reagents should be cleaned up immediately. Observe all federal, state and local environmental regulations when disposing of wastes.

Precautions
1. This product is for In Vitro Diagnostic Use.
2. This product should only be used by appropriately trained personnel.
3. 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.
4. 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 must be taken from the same foil pouch.
5. To avoid reagent contamination, only use new or clean plastic / glassware. Never return unused reagents to the bottles.
6. Do not leave reagent bottles uncapped; any resulting evaporation or contamination will lead to inconsistent results.
7. TMB chromogen must not be exposed to light or water.
8. Microbiologically contaminated, haemolysed or lipaemic serum and specimens containing particulate matter should not be used.
9. 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.
10. 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.
11. All equipment used must be calibrated and maintained according to the manufacturer’s instruction.

Storage Conditions
1. The kit should be stored at 2-8°C and should not be frozen. Inappropriate storage temperatures will affect the results.
2. Diluted wash buffer is stable for 1 week at 2-8°C.
3. The expiry date of the kit is shown on the outer label.

Specimen Collection
1. Blood samples should be collected by venepuncture allowed to clot naturally and the serum separated.
2. 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.
3. Repeated thawing and freezing should be avoided.
4. Serum samples should not be heat-inactivated, as this may give false positive results.

Procedure
Materials provided
- Instruction leaflet: Giving full assay details.
- QC Certificate: Indicating the expected performance of the batch.
- Human Transglutaminase Coated Wells: 12 breakapart 8 well strips coated with recombinant tTG. Each plate is packaged in a re-sealable foil bag containing two desiccant pouches.
- HRP Sample Diluent: 2 bottles containing 50mL of buffer for sample dilution. Colored pink, ready to use.
- HRP Wash Concentrate: 1 bottle containing 25mL of a 40-fold concentrated buffer for washing the wells.
- R h-tTG IgA Calibration: 5 bottles, each containing 1.2mL of diluted human serum, with the following concentrations of anti-tTG IgA autoantibody: 100, 33.3, 11.1, 3.7, 1.23 U/mL. Ready to use.
- R h-tTG IgA Positive Control: 1 bottle containing 1.2mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
- ELISA Negative Control: 1 bottle containing 1.2mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
- HRP R h-tTG IgA Conjugate: 1 bottle containing 10mL of purified peroxidase labelled antibody to human IgA. Colored yellow. Ready to use.
- TMB Chromogen: 1 bottle containing 10mL TMB chromogen. Ready to use.
- Stop Solution HRP: 1 bottle containing 10mL of 0.344M Sulfuric Acid. Ready to use.

Additional materials required but not provided
- 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 1000, 100 & 10µL
- Multichannel pipette: Recommended for dispensing 100µL volumes of conjugate, substrate and stop solution.
- Glass/plastic tubes: For sample dilution
Method

Before you start
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. Note: The kits may be maintained at room temperature for up to 1 week.
2. Kit components
   Gently mix each kit component before use.
3. Wash buffer dilution
   Add 25mL of the wash buffer concentrate to 975mL of distilled water (1 in 40 dilution) into a clean container and mix. Note: Diluted wash buffer is stable for 1 week at 2-8°C, 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 (1:101) and mix. Note: 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. Note: 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.

Assay procedure

Maintain the same dispensing sequence throughout the assay.
1. Sample addition
   Dispense 100µL of each calibrator, control and diluted (1:101) 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 200-300µL 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. Flick out the contents of the plate into a sink.
   b. Tap the wells dry on absorbent paper.
   c. Fill each well with 200-300µL of wash buffer using a multichannel pipette.
   d. Gently shake the plate on a flat surface.
   e. Repeat a-d twice.
   f. Repeat a and b.
3. Conjugate addition
   Dispense 100µL of conjugate into each well, blot the top of the wells with a tissue to remove any splashes. Note: To avoid contamination, never return excess conjugate to the reagent bottle. Incubate at room temperature for 30 minutes.
4. Washing
   Repeat step 2.
5. Chromogen (TMB) addition
   Dispense 100µL of TMB chromogen 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.
6. Stopping
   Dispense 100µL of stop solution into each well. This causes a change in colour from blue to yellow.
7. Optical density measurement
   Read the optical density (OD) of each well at 450nm on a microplate reader, within 30 minutes of stopping the reaction.

Quality Control

1. Quality control
   In order for an assay to be valid, all the following criteria must be met:
   • Calibrators and positive and negative controls must be included in each run.
   • The values obtained for the positive and negative controls should be in the ranges specified on the QC Certificate.
   • The curve shape should be similar to the calibration curve, shown on the QC Certificate.
   If the above criteria are not met, the assay is invalid and the test should be repeated.
2. Calculate 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%.
3. Plot calibration curve
   The calibration curve can be plotted either automatically or manually as follows by plotting the anti-ITG IgA autoantibody concentration on the log scale 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).
4. **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.

5. **Calculation of the control values**
Read the level of the anti-tTG IgA autoantibody from the calibration curve. The values should fall within the
ranges given on the QC Certificate.

6. **Calculation of autoantibody levels in diluted samples**
Read the level of the anti-tTG IgA autoantibody 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 1:101 sample
dilution, therefore no further conversion is required.

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

**Limitations of the Procedure**
1. This kit is used to aid diagnosis only. A positive result suggests certain diseases that must be
confirmed by clinical findings and other serological tests.
2. The results obtained from this assay are not diagnostic proof of the presence or absence of disease.
3. The use of this assay and normal range (result interpretation) has not been established for
paediatric samples.
4. A negative result may be due to IgA deficiency and does not rule out coeliac disease.

**Expected Values**
The normal range was determined on serum from 200 normal adult blood donors. One of these samples was
confirmed to contain anti tTG 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.

<table>
<thead>
<tr>
<th>RESULT INTERPRETATION</th>
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<tbody>
<tr>
<td>Negative</td>
<td>&lt;4.0  U/mL</td>
</tr>
<tr>
<td>Weak Positive</td>
<td>4 - 10 U/mL</td>
</tr>
<tr>
<td>Positive</td>
<td>&gt;10 U/mL</td>
</tr>
</tbody>
</table>

**Performance Characteristics**

**Precision**
The intra-assay precision was measured using six samples tested within the range of the calibration curve. The %
CV for each sample is given below:

<table>
<thead>
<tr>
<th>INTRA-ASSAY PRECISION</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n=20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (U/mL)</td>
<td>% CV</td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Sample 2</td>
<td>5.1</td>
<td>3.9</td>
</tr>
<tr>
<td>Sample 3</td>
<td>15.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Sample 4</td>
<td>21.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Sample 5</td>
<td>29.4</td>
<td>5.5</td>
</tr>
<tr>
<td>Sample 6</td>
<td>55.8</td>
<td>3.3</td>
</tr>
</tbody>
</table>

The inter-assay precision was measured using six samples tested in duplicate six times for three days. The % CV
for each sample is given below:

<table>
<thead>
<tr>
<th>INTER-ASSAY PRECISION</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>n=6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (U/mL)</td>
<td>% CV</td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>3.2</td>
<td>4.5</td>
</tr>
<tr>
<td>Sample 2</td>
<td>5.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Sample 3</td>
<td>17.2</td>
<td>6.8</td>
</tr>
<tr>
<td>Sample 4</td>
<td>25.2</td>
<td>7.1</td>
</tr>
<tr>
<td>Sample 5</td>
<td>30.5</td>
<td>6.9</td>
</tr>
<tr>
<td>Sample 6</td>
<td>74.0</td>
<td>6.7</td>
</tr>
</tbody>
</table>

**Analytical Sensitivity**
Assay sensitivity of 1.23 U/mL was confirmed by assaying two samples in multiple replicates with values 1.5 and
2.5 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).

**Measuring Range**
The measuring range of the assay is 1.23 - 100 U/mL.
Normal Range
Anti-tTG IgA autoantibodies levels were measured in serum from 200 normal adult blood donors. Based on the guideline result interpretation, 0.5% (1/200) were positive for tTG IgA autoantibodies. This sample also tested positive in an alternative tTG IgA EIA kit.

In addition, 39/40 samples from patients with Crohn’s disease (n=21) and ulcerative colitis (n=19) tested negative in the anti-tTG IgA assay. The single positive Crohn’s disease sample was confirmed positive by anti-IgA endomysial IFA.

Relative Specificity, Sensitivity, Agreement
The relative specificity, sensitivity and agreement has been determined against an anti-IgA endomysial IFA kit using 106 test samples from biopsy confirmed Coeliac/non-Coeliac patients.

<table>
<thead>
<tr>
<th>BINDAZYME™ Anti-transglutaminase IgA</th>
<th>Anti-IgA Endomysial IFA</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td>53</td>
</tr>
<tr>
<td>-</td>
<td>49</td>
</tr>
<tr>
<td>Relative sensitivity</td>
<td>98.2 %</td>
</tr>
<tr>
<td>Relative specificity</td>
<td>94.2%</td>
</tr>
<tr>
<td>Relative agreement</td>
<td>96.2%</td>
</tr>
</tbody>
</table>

a. 3/3 EIA positive / IFA negative samples were from biopsy confirmed Coeliac patients.
b. Sample weakly positive on IFA

Interfering Substances
A range of interfering substances has been spiked into anti-tTG IgA negative and positive samples, which have then been subsequently assayed. The method used to check these substances was based on the Interference Check A plus™ -Kokusai Shiyaku, Japan.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin F (Free)</td>
<td>19.3mg/dL</td>
</tr>
<tr>
<td>Bilirubin C (Conjugate)</td>
<td>19.9mg/dL</td>
</tr>
<tr>
<td>Haemolysed Haemoglobin</td>
<td>485mg/dL</td>
</tr>
<tr>
<td>Chyle</td>
<td>1550 Units</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>45 IU/mL</td>
</tr>
</tbody>
</table>

No interference by these substances was observed in either sample tested.

Assay Linearity
Three samples have been tested for linearity across the calibration range, the regression coefficient R^2 was greater than 0.997 when comparing the actual to the expected values in U/mL, with the mean recovery of 99%.

Prozone studies
Three positive samples were diluted 1:6.25 in sample diluent (normal dilution 1:101) to assess possible prozone effects. All samples diluted out appropriately, no false negative values were observed at the lowest sample dilutions, thus no prozone was observed.
**Summary of procedure**

1. Add 100µL of each calibrator, control and 1:101 diluted sample to the appropriate wells.
   - **Incubate for 30 minutes.**
   - Wash.

2. Add 100µL of conjugate to each well.
   - **Incubate for 30 minutes.**
   - Wash.

3. Add 100µL of substrate to each well.
   - **Incubate for 30 minutes.**

4. Add 100µL of stop solution to each well.
   - Measure the absorbance at 450nm.
References

Manufactured By:
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