M1551/ November 2007

**ELISA**

PeliClass human IgG subclass kit

ELISA kit for quantitative determination of human IgG subclasses in serum (en)

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I. INTRODUCTION

Human IgG comprises four subclasses: IgG1, IgG2, IgG3 and IgG4. The biochemical characteristics of the IgG subclasses have been described extensively (1-5). The differences between IgG subclasses are reflected in several biologically important functions such as antigen recognition, complement activation and binding to cell surface receptors. Many studies have revealed that abnormalities in serum levels of IgG subclasses may be associated with various disease states. Especially the association of selective IgG2 subclass deficiency with increased susceptibility to viral or bacterial infections has been amply documented (4, 5). Low serum levels of IgG2 or IgG3 have been reported in patients with recurrent upper and lower respiratory tract infections. Others have found an association between very low IgG4 serum concentrations and recurrent sino-pulmonary infections (6). Abnormalities in the serum levels of IgG subclasses have also been observed in autoimmune diseases, neurological disorders and in HIV infections (4).

II. TECHNICAL PRINCIPLE

The PeliClass human subclass ELISA kit is a 'sandwich'-type enzyme immunoassay. The kit contains microwell strips coated with highly avid monoclonal antibodies, each specific for one of the human IgG subclasses. Test samples, calibrator- and control sera are incubated in the respective wells. The IgG subclass to be determined will bind to the solid phase and non-bound IgG is removed by washing. Next, peroxidase-conjugated anti-human IgG antiserum is added to each well and non-bound conjugate is removed by washing. After incubation with substrate solution (ABTS) and H₂O₂, the reaction is stopped with an acid buffer. The green colored reaction product is measured spectrophotometrically and the concentration of IgG subclass in the test sample calculated relative to the values of a reference curve. IgG subclass control serum is assayed to check the validity of the calibration curves and the accuracy of the IgG subclass determinations.

The IgG subclass levels in the calibrator(s) were determined using a calibrator derived from the WHO 67/97 reference preparation. The recommended target values of 5.0 g/L for IgG1, 2.6 g/L for IgG2, 0.4 g/L for IgG3 and 0.5 g/L for IgG4 were used (7).

III. STORAGE AND STABILITY

The PeliClass human subclass ELISA kit should be stored upright at 2-8 °C. It can be used until the expiration date shown on the label. For all components stability after opening is 1 week when stored at 2-8 °C. Transport conditions may differ from storage conditions.

IV. CONTENTS OF THE KIT

See the Table at the beginning of this package insert. The PeliClass human subclass ELISA kit contains sufficient reagents for 48 tests for each subclass, including calibrators, controls and blank. The monoclonal antibodies were purified from tissue culture medium, using column chromatography (ion exchange and affinity chromatography). The calibrator and control are liquid human sera.

V. ADDITIONAL MATERIALS REQUIRED

- Distilled water for dilution of wash- dilution- and substrate buffers.
- Pipetting devices for accurate delivery of volumes.
- An incubator (37 ± 2°C).
- A standard ELISA washer or a 500 mL plastic squirt bottle for automatic or manual washing of the strips.
- A standard ELISA reader for measuring absorbance at 414 nm or 405 nm.
- Log linear paper.

VI. TEST SAMPLE HANDLING

Only serum samples should be tested. The samples should be as fresh as possible or stored frozen. The samples should be manually diluted before use (see VII ASSAY PROTOCOL).

VII. ASSAY PROTOCOL

1. MICROTITREPLATE

The PeliClass human IgG subclass ELISA kit provides the flexibility to use partial plates on separate occasions. Before opening the plastic pouch, determine the number of strips required to test the desired number of samples plus 14 wells needed for running calibrators, controls and blanks in duplicate. Remove strips that will not be used from the plate-frame and re-pack them in the plastic pouch containing the desiccant and store at 2-8°C.

2. BUFFER PREPARATIONS

Wash buffer: Prepare the wash buffer by adding the total content of the bottle of the wash buffer concentrate to 950 mL distilled water. The diluted wash buffer must be stored at 2-8°C and remains stable for 1 week.

Note: The concentrated buffer may contain salt crystals. Before preparing the working-strength buffer, warm the concentrated buffer BRIEFLY to 37°C to dissolve the crystals.

Dilution buffer: Calculate the quantity of dilution buffer required (approximately 2 mL undiluted buffer per microwell strip) and prepare a working-strength solution by diluting the buffer 10 fold in distilled water.

3. PREPARATION OF THE CALIBRATOR AND CONTROL SERA

For concentrations see Table 2 and 3 of the enclosed information leaflet.

Calibrator: (See tabel 1 of the enclosed information leaflet) Label one 10 mL tube with ‘1:500’ and eight 3 mL tubes with ‘Cal1 to Cal8’ respectively.

Pipette 4.99 mL of dilution buffer into the 10 mL tube and add 10 µL of calibrator serum (initial dilution 1:500). Pipette 1.9 mL of dilution buffer in the tube labeled ‘Cal1’ and 1.0 mL into the tubes labeled ‘Cal2 - Cal8’.

Pipette 100 µL 1:500 diluted calibrator to the tube Cal1, and make from this tube seven 2-fold serial dilutions by adding 1.0 mL from the previous dilution to the next ‘Cal’ tube. Select for each IgG subclass the series of calibrator dilutions: IgG1 1:80,000 -1:1,280,000 IgG2, IgG3 and IgG4 1:10,000 -1:160,000.

Control: Label one tube with ‘1:500’, and two 3 mL tubes with ‘1:30,000’ (for IgG2, 3, 4) and ‘1:240,000’ (for IgG1) respectively.

Pipette in the tube labeled with 1:500 → 4.99 mL dilution buffer and 10 µL control serum.
Add 50 µL of stop solution to all wells. Incubate for 30 minutes at room temperature (18-25°C). Gently agitate by tapping the edge of the microtitreplate for a few seconds to mix the contents of each well. Add 100 µL of substrate solution to all wells. 

substrate buffer (2 mL substrate stock solution + 18 mL distilled water). 

Add the equivalents of 200 µL hydrogen peroxide stock solution and 400 µL ABTS stock solution to 20 mL of working strength 

Calculate the amount of substrate solution (approximately 0.9 mL per microwell strip will be needed). Aspirate supernatant from the wells and wash the plate as described in point 5. Incubate for 1 hour at 37°C. Cover the plate with adhesive seal, gently agitate by tapping the edge for a few seconds to mix the contents of each well. Add 100 µL of the 1:2000 dilution to the anti-IgG3 microwell strips; 100 µL of the 1:3000 dilution to the anti-IgG2 microwell strips; Add: 100 µL of the 1:500 dilution to the anti-IgG1 microwell strips; 1:100 by pipetting 3.5 mL of the 1:500 dilution to 6.0 mL dilution buffer. Dilute the conjugate 1:500 by pipetting 30 µL of the conjugate to 14.97 mL dilution buffer. Dilute the conjugate further to: 1:200 by pipetting 2.0 mL of the 1:500 dilution to 6.0 mL dilution buffer. 1:300 by pipetting 1.2 mL of the 1:500 dilution to 6.0 mL dilution buffer. 

Dilute the conjugate 1:500 by pipetting 30 µL of the conjugate to 14.97 mL dilution buffer. Dilute the conjugate further to: 

1:200 by pipetting 2.0 mL of the 1:500 dilution to 6.0 mL dilution buffer. 1:300 by pipetting 1.2 mL of the 1:500 dilution to 6.0 mL dilution buffer. 

Aspirate supernatant from the wells and wash the plate as described in point 5. Incubate for 1 hour at 37°C. Cover plate with adhesive seal, gently agitate by tapping the edge of the microtitreplate for a few seconds to mix contents of each well. Add 100 µL of the diluted calibrators, controls, samples and blanks into the appropriate wells. 

Just before washing, prepare next incubation reagent as described in point 8. Just before washing, prepare next incubation reagent as described in point 5. 12. PLATE READ-OUT Read within 1 hour at 414 (preferably) or 405 nm in an ELISA reader. 

\[ \text{Absorbance at 414 (preferably) or 405 nm in an ELISA reader.} \]

\[ \text{Reference ranges (g/L) for IgG subclasses in serum samples of healthy Caucasian individuals (8). For other populations separate} \]

\[ \text{reference ranges should be obtained.} \]

\[ \begin{array}{|c|c|c|c|}
\hline
\text{Age} & \text{IgG1} & \text{IgG2} & \text{IgG3} \\
0 - 1 \text{ mnth} & 2.4 - 10.6 & 0.87 - 4.1 & 0.14 - 0.55 \\
1 - 4 \text{ mnth} & 1.8 - 6.7 & 0.38 - 2.1 & 0.14 - 0.70 <0.03 - 0.36 \\
4 - 6 \text{ mnth} & 1.8 - 7.0 & 0.34 - 2.1 & 0.15 - 0.80 <0.03 - 0.23 \\
6 - 12 \text{ mnth} & 2.0 - 7.7 & 0.34 - 2.3 & 0.15 - 0.97 <0.03 - 0.43 \\
1 - 2 \text{ yr} & 2.5 - 8.2 & 0.38 - 2.4 & 0.15 - 1.07 <0.03 - 0.62 \\
1 \frac{1}{2} - 2 \text{ yr} & 2.9 - 8.5 & 0.45 - 2.6 & 0.15 - 1.13 <0.03 - 0.79 \\
2 - 3 \text{ yr} & 3.2 - 9.0 & 0.52 - 2.8 & 0.14 - 1.20 <0.03 - 1.06 \\
3 - 4 \text{ yr} & 3.5 - 9.4 & 0.63 - 3.0 & 0.13 - 1.26 <0.03 - 1.27 \\
4 - 6 \text{ yr} & 3.7 - 10.0 & 0.72 - 3.4 & 0.13 - 1.33 <0.03 - 1.58 \\
6 - 9 \text{ yr} & 4.0 - 10.8 & 0.85 - 4.1 & 0.13 - 1.42 <0.03 - 1.89 \\
9 - 12 \text{ yr} & 4.0 - 11.5 & 0.98 - 4.8 & 0.15 - 1.49 0.03 - 2.10 \\
12 - 18 \text{ yr} & 4.7 - 12.8 & 1.06 - 6.1 & 0.18 - 1.63 0.04 - 2.30 \\
> 18 \text{ yr} & 4.9 - 11.4 & 1.50 - 6.4 & 0.20 - 1.10 0.08 - 1.40 \\
\hline
\end{array} \]

This table provides reference ranges for IgG subclasses in serum samples of healthy Caucasian individuals. For other populations, separate reference ranges should be obtained.
XI. SPECIFIC PERFORMANCE CHARACTERISTICS

a Reproducibility

<table>
<thead>
<tr>
<th>IgG subclass concentration</th>
<th>IgG1</th>
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<td>3</td>
<td>4</td>
<td>2</td>
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<tr>
<td>inter-assay variation (%)</td>
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<td>2</td>
<td>7</td>
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</table>

b Comparison of PeliClass human IgG subclass kit vs. Mancini.

The IgG1, IgG2, IgG3 and IgG4 concentrations in sera were determined by an ELISA assay and compared with the corresponding values found in Mancini. The following correlations were established:

<table>
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<tr>
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<th>correlation</th>
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<td>0.97</td>
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<tr>
<td>IgG2</td>
<td>Y = 1.01X – 0.17</td>
<td>0.93</td>
</tr>
<tr>
<td>IgG3</td>
<td>Y = 0.91X + 0.00</td>
<td>0.99</td>
</tr>
<tr>
<td>IgG4</td>
<td>Y = 0.96X – 0.03</td>
<td>0.98</td>
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Note:
The values cited for specific performance characteristics of the test represent typical results and are not to be viewed as specifications for this kit.

XII. RESTRICTIONS

1. User should be trained and familiar with ELISA assays and test procedure.
2. Grossly haemolysed or lipaemic samples should not be used. Unexpected results may be obtained for samples containing rheumatoid factor, high bilirubin levels, or other circulating immune complexes. These samples should be analysed by other method.
3. Out of range samples, e.g. in case of paraproteins, should be repeated using different dilutions.
4. The finding of a decreased level of one of the IgG subclasses can never provide a definite diagnosis, but should rather be considered as an indication of a disturbance of the immune system, requiring further diagnostic investigation.
5. Control serum should always be used to check the validity of the calibration curves. When the control is out of range, the results of the test samples are not reliable. The test should be repeated.
6. Reagents from different batches are not interchangeable.
7. Rests of reagents (e.g. dead volume) should not be mixed with contents of freshly opened vials.
8. Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
9. Although the human calibrator and control sera have been tested for the markers of specific disease transmitting agents in accordance with current EU guidelines to GMP and found to be nonreactive, all components of human origin should be considered as potentially infectious.
10. Preservative: Thiomersal® 0,001%.
11. Use new plate seals for each incubation/fixation step in the ELISA-experiment to avoid cross contamination. Do not use aluminium foil.
12. Use disposable pipette tips for each transfer to avoid cross contamination.
13. Each time the kit is used, fresh dilutions of calibrators, conjugate and buffers should be made.
14. Do not use other reagents and microtitre strips than those supplied with the kit.
15. Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied buffers.
16. The waste-disposal should be performed according to your laboratory regulations.

XIII. REFERENCES