Enzyme immunoassay for the quantitative determination of the pregnancy associated plasma protein-A (PAPP-A) in human serum during the first trimester of pregnancy.

40-056-205071 (96 tests – 12 x 8 wells)

Research use only.

1. INTENDED USE

This PAPP-A ELISA is an enzyme immunoassay for the determination of the pregnancy associated plasma protein-A (PAPP-A) in human serum during the first trimester of pregnancy (at 9 to 13 weeks) for the screening of Down’s syndrome and other chromosomal abnormalities. This kit, designed for evaluating the risk of trisomy 21, can be used in combination with the T21 risk analysis software which must be validated by the end-user.

2. INTRODUCTION

PAPP-A (Pregnancy associated plasma protein A) is a large glycoprotein (720 KDa) which is produced by the developing placenta during pregnancy and released into maternal circulation. The circulating PAPP-A is a disulfide bonded complex consisting of two PAPP-A subunits and two subunits of a glycosylated form (50 – 90 KDa) of eosinophil major basic protein (proMBP). The placenta is likely to be the main source of circulating PAPP-A/proMBP in pregnancy. The PAPP-A/proMBP levels appear to increase with gestational age until delivery.

Maternal PAPP-A assessment between 9 and 13 weeks of pregnancy has been reported to be an useful marker for the screening of Down’s syndrome and other chromosomal abnormalities.

Meanwhile, a new approach has been introduced by several authors based on nuchal translucency measurement of the fetal neck. When combining biochemical parameters (PAPP-A, Free Beta hCG) maternal age related risk and nuchal translucency measurement, several authors have reported detection rates for Donw’s syndrome of 80 to 90% at 5% false positivity rate.

3. TEST PRINCIPLE

The PAPP-A ELISA is a solid phase enzyme linked immunosorbent assay (ELISA) based on the sandwich principle. The microtiter wells are coated with a rabbit immunoglobulin anti-PAPP-A.

PAPP-A present in the sample, binds to the anti-PAPP-A antibody coated onto the microtiter plate. After removal of unreacted material by a washing step, an antibody anti-PAPP-A labelled with horseradish peroxidase is added to the wells.

So, the following complex is formed:

Rabbit IgG anti-PAPP-A + PAPP-A +
Antibody Anti-PAPP-A peroxidase conjugate.

After a second incubation followed by a washing step, the immuno-complex is detected by reaction with TMB substrate and the development of a blue colour which changes into yellow by stopping the enzymatic reaction with sulfuric acid.

The intensity of this colour is directly proportional to the amount of PAPP-A in the sample. Absorbance at 450 nm is read using an ELISA microtiter plate reader.

4. REAGENTS AND MATERIAL SUPPLIED

When stored at 2 – 8 °C, all unopened reagents of the kit will retain reactivity until expiry date. Do not use reagents beyond this date.

   Immediately, after removal of strips, the remaining strips should be resealed in the outer bag along with the desiccant and stored at 2- 8°C. It is important to ensure the desiccant remains in the bag.

2. PAPP-A calibrators: 6 vials calibrators supplied in BSA standard matrix base. Preservative: thimerosal 0.04 %. Store at 2 – 8°C. Reconstitute the lyophilized calibrators with fresh and germ free distilled water as follow: add 1.2 ml of H2O to each vial, mix well. It is recommended to reconstitute the calibrators in advance; they should be completely resuspended before use. The reconstituted calibrators are stable one month at 2-8°C.
   Check on the box of each lot for the accurate values of the calibrators and controls.

3. Enzyme conjugate: 1 vial (0.25 ml) of antibody anti-PAPP-A conjugated to horseradish peroxidase (HRPO) 100 x concentrated. Preservative: thimerosal 0.02 %. Dilute 100x concentrated enzyme conjugate with dilution buffer. The enzyme conjugate has to be prepared freshly 30 min before use. Dilute only the quantity required for each test run.
   Store concentrated at 2 – 8°C.

4. Control serum 1 & 2: one set of 2 vials, each containing a lyophilized human serum. Preservative: NaCl 0.05 %) with two different amounts of PAPP-A (low and medium concentration). Reconstitute each vial with 0.8 ml of H2O, mix well.
   After reconstitution the controls are stable one month at 2-8°C.
   For the expected value refer to the box label. Store lyophilized at 2-8°C.

5. Washing solution: 1 vial (100 ml) of 15 x concentrated buffer with Tween 20. Preservative: thimerosal 0.01 %. Bring the vial content to 100 + 1400 = 1500 ml (final volume) with fresh and germ free distilled water. The diluted washing solution is stable for 1 week at 2-8°C.
   Store concentrated at 2 – 8°C.

   Store at 2 – 8°C.

7. Blocking reagent: 1 vial (15ml) of 0.5 M H2SO4. Ready for use.
Irritant agent.
Stable at 2 – 8°C

8. Dilution buffer: 1 vial (35 ml) of phosphate buffer with BSA. Preservative: Thimerosal. Ready to use
Store at 2 – 8°C

Sealing tape Strip holder Instruction leaflet

KIT REAGENTS

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity</th>
<th>Physical state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wells</td>
<td>96</td>
<td>Ready for use</td>
</tr>
<tr>
<td>Calibrators 0 – 5</td>
<td>6 x 1.2 ml</td>
<td>Lyophilised</td>
</tr>
<tr>
<td>Control Sera</td>
<td>2 x 0.8 ml</td>
<td>Lyophilised</td>
</tr>
<tr>
<td>HRP conjugate</td>
<td>1 x 0.25 ml</td>
<td>Concentrated 100 x</td>
</tr>
<tr>
<td>Washing Solution</td>
<td>1 x 100 ml</td>
<td>Concentrated 15 x</td>
</tr>
<tr>
<td>TMB Substrate solution</td>
<td>1 x 13 ml</td>
<td>Ready for use</td>
</tr>
<tr>
<td>Blocking Reagent</td>
<td>1 x 15 ml</td>
<td>Ready for use</td>
</tr>
<tr>
<td>Dilution buffer</td>
<td>1 x 35 ml</td>
<td>Ready for use</td>
</tr>
</tbody>
</table>

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450/620nm
- Incubator 37°C
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Deionised or (freshly) distilled water
- Disposable tubes
- Timer
- Risk analysis software

6. PRECAUTIONS

1. Use this kit for research use only.
2. Keep all reagents at (2-8°C) in closed containers when not in use.
3. Ensure that all reagents are equilibrated to 18-25°C before use.
4. Do not use any solutions which have become turbid.
5. Do not use reagents after expiry date stated on the label.
6. Do not interchange reagent vials and their screw caps to avoid cross-contamination.
7. Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.
8. All components of human origin have been tested for HBs Ag, anti-HIV and anti-HCV antibodies and have been found to be non-reactive. However, all patient specimens and kit components should still be regarded and handled as potentially infectious.
9. The stop solution contains 1 mol/l Sulfuric Acid. Caution: Corrosive. (See MSDS)
   - Buffers and calibrators contain thimerosal as a preservative. (See MSDS)
10. The controls contain NaN₃ 0.05% as preservative (See MSDS). For information on hazardous substances included in the kit please refer to Material Safety Data sheets (available upon request).
11. Do not pipette with mouth.
12. The general purpose reagents washing/dilution buffers and stopping solution are interchangeable between different lots while all other reagents are specific for the individual package lot and must not be interchanged with other lots.
13. Do not use reagents from other manufacturers along with the kit reagents for a given test run.
14. Use a clean, fresh, disposable pipette tip for each reagent or specimen manipulation.
15. Do not freeze the kits.

7. SPECIMEN COLLECTION

Use serum samples.
The clotted samples must be centrifuged as soon as possible. No special pretreatment of the sample is necessary. Do not use hemolyzed or lipemic specimens.

Storage: serum samples can be kept at 2-8°C for maximum 48 h. For longer periods store at -20°C or lower. Avoid multiple freeze-thaw cycles for any specimen.

8. TEST PROCEDURE

1. Reliability of results

The test must be performed exactly as per the manufacturer’s instructions for use. Moreover, the user must strictly adhere to the rules of Good Laboratory Practice or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test. Note: The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure.

2. PAPP-A determination

Bring all reagents, samples, calibrators and controls to room temperature before use.
Prior to starting the assay, the distribution and identification plan for all specimens and calibrators should be carefully established.
Select required number of microtitre strips and place in the strip holder. Allow 1 well for the substrate blank and 6 wells for the calibrators but it is recommended to perform the standard curve in duplicate.
Step 1
1. Leave well A1 for substrate blank. Dispense calibrators in the following order:

- 100 µl calibrator C0 - well B1
- 100 µl calibrator C1 - well C1
- 100 µl calibrator C2 - well D1
- 100 µl calibrator C3 - well E1
- 100 µl calibrator C4 - well F1
- 100 µl calibrator C5 - well G1

Dilute patient's sera and controls directly in the wells: 10 µl of serum + 200 µl of dilution buffer and mix gently.

**Reconstituted calibrators do not require dilution**
2. Cover wells with foil or adhesive film.
3. Incubate 60 min at 37 °C
4. Aspirate off contents of wells and add to each well 4 times 300 µl of washing buffer and aspirate off again.
   - If an automatic washer is used, primarily wash with **working washing solution** and repeat 4 times.
   - Ensure the washer fills all wells completely and aspirates off efficiently between washing steps (remaining liquid < 15 µl)!
   - At the end carefully remove remaining fluid by tapping the strips on tissue paper prior to next step.

Step 2
1. Dispense 100 µl of **prediluted anti-PAPP-A peroxidase conjugate** (see to reagents and material supplied) in all wells except well A1
2. Cover wells with foil or adhesive film.
3. Incubate 60 min at 37°C
4. Aspirate off contents of wells and add to each well 4 times 300 µl of washing buffer and aspirate off again.
   - If an automatic washer is used, primarily wash with **working washing solution** and repeat 4 times.
   - Ensure the washer fills all wells completely and aspirates off efficiently between washing steps (remaining liquid < 15 µl)!
   - At the end carefully remove remaining fluid by tapping the strips on tissue paper prior to next step.

Step 3
1. Dispense 100 µl of TMB solution to all wells.
2. Incubate 10 min at room temperature (15-30°C) in the dark
3. Stop the enzymatic reaction by addition of 100 µl of **stopping solution** to all wells.
5. Measure the absorbance at 450 nm (A450).
   - **Note:** Dual wavelength reading using 620 nm or 690 nm as reference wavelength is recommended but not compulsory.
   - The absorbance should be read within 15 min.

9. INTERPRETATION OF RESULTS

**A. Standard curve**

Automatically: select semi-log Cubic spline graph on the microplate reader

Manually: For each parameter, draw a graph as follows:
1. **ORDINATES:** measured optical density value for each Calibrator
2. **ABSCISSAE:** decimal logarithm of concentrations of each Calibrator.

Plot optical density values of the samples on the curve

O.D. = f (Log concentration C)

Read in abscissae concentration logarithm (X) Calculate C (concentration) : $C = 10^X$

**APPROXIMATE VALUES**

<table>
<thead>
<tr>
<th>PAPP-A (mg/L)</th>
<th>C0</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td></td>
<td></td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td></td>
<td></td>
<td></td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean (mg/L)</th>
<th>Expected range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>18.45 [13.84 - 23.06]</td>
</tr>
<tr>
<td>Control 2</td>
<td>38.63 [28.97 - 48.28]</td>
</tr>
</tbody>
</table>

**CAUTION**
The samples showing a concentration exceeding that of the calibrator C5 should be prediluted in dilution buffer and tested again. The concentration thus obtained has to be multiplied by the dilution factor.

**B. Validation of the test**
The test may be considered valid provided the following criteria are met:
1. The substrate blank in well A1 appears colourless to the eye
2. Absorbance value
   - C0 = 0.2
   - C5 = 1.5
10. EXPECTED VALUES

<table>
<thead>
<tr>
<th>Weeks of gestation</th>
<th>Percentile 5</th>
<th>Percentile 50</th>
<th>Percentile 95</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>3.61</td>
<td>18.11</td>
<td>91.00</td>
<td>109</td>
</tr>
<tr>
<td>10</td>
<td>4.92</td>
<td>24.73</td>
<td>124.23</td>
<td>122</td>
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<td>11</td>
<td>6.46</td>
<td>32.45</td>
<td>163.00</td>
<td>151</td>
</tr>
<tr>
<td>12</td>
<td>8.25</td>
<td>41.45</td>
<td>208.22</td>
<td>165</td>
</tr>
<tr>
<td>13</td>
<td>10.34</td>
<td>51.94</td>
<td>260.95</td>
<td>184</td>
</tr>
</tbody>
</table>

Those normal ranges should be used as a guideline only. Since ethnic and geographical differences of PAPP-A levels in normal pregnancies may exist, it is recommended that each laboratory establishes the normal range (medians for each gestational age) for its own patient population.

11. PERFORMANCES OF THE ASSAY

SPECIFICITY: No cross-reaction was found for Free ß-hCG, AFP, hPL and FSH, respectively, using concentrations far above physiological levels.

SENSITIVITY: The sensitivity was calculated based upon the standard curve and expressed as the minimal dose showing a significant difference from the Zero Standard (mean value + 3 S.D.) This dose is 1.5 mg/L.

PRECISION: Precision was evaluated upon intra- and inter-assay variability, in 3 sera at different PAPP-A concentrations (mg/L).

Intra-Assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mean mg/L</th>
<th>CV %</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.22</td>
<td>7.56</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>23.07</td>
<td>4.62</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>40.76</td>
<td>4.07</td>
<td>32</td>
</tr>
</tbody>
</table>

Inter-Assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mean mg/L</th>
<th>CV %</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.6</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>5.76</td>
<td>3.22</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>28.12</td>
<td>2.4</td>
<td>3</td>
</tr>
</tbody>
</table>

ACCURACY: Accuracy of the method has been checked as follows: Recovery test: 3 samples containing different levels of endogenous PAPP-A were spiked with different amounts of PAPP-A (mg/L).

<table>
<thead>
<tr>
<th>Endogenous</th>
<th>Added</th>
<th>Expected</th>
<th>Measured</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.31</td>
<td>27.6</td>
<td>35.91</td>
<td>31.61</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>13.2</td>
<td>21.51</td>
<td>20.12</td>
<td>93.5</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>13.71</td>
<td>14.26</td>
<td>104</td>
</tr>
<tr>
<td>15.7</td>
<td>27.6</td>
<td>43.3</td>
<td>38.49</td>
<td>88.9</td>
</tr>
<tr>
<td></td>
<td>13.2</td>
<td>28.9</td>
<td>26.03</td>
<td>90.1</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>21.1</td>
<td>20.24</td>
<td>95.9</td>
</tr>
<tr>
<td>26.05</td>
<td>12.5</td>
<td>38.55</td>
<td>32.98</td>
<td>85.6</td>
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<tr>
<td></td>
<td>6.25</td>
<td>32.3</td>
<td>30.32</td>
<td>93.9</td>
</tr>
<tr>
<td></td>
<td>1.56</td>
<td>27.61</td>
<td>27.23</td>
<td>98.6</td>
</tr>
</tbody>
</table>

Parallelism Test: 3 serum samples containing a low, medium and high endogenous PAPP-A were diluted serially with the Zero standard. The obtained values are presented in the table.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Dilutions</th>
<th>Expected (mg/L)</th>
<th>Measured</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/2</td>
<td>4.57</td>
<td>4.32</td>
<td>94.6</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>2.28</td>
<td>2.47</td>
<td>108.2</td>
</tr>
<tr>
<td>2</td>
<td>1/2</td>
<td>14.05</td>
<td>16.08</td>
<td>114.5</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>7.02</td>
<td>6.98</td>
<td>99.3</td>
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<tr>
<td></td>
<td>1/8</td>
<td>3.51</td>
<td>3.5</td>
<td>99.6</td>
</tr>
<tr>
<td>3</td>
<td>1/2</td>
<td>21</td>
<td>24.54</td>
<td>116.9</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>10.5</td>
<td>11.52</td>
<td>109.7</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>5.25</td>
<td>5.66</td>
<td>107.8</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>2.63</td>
<td>2.97</td>
<td>113</td>
</tr>
</tbody>
</table>
12. METHOD COMPARISON

The performance of PAPP-A ELISA test has been assessed by determination of PAPP-A concentration in 51 samples in comparison with another commercially available kit. The correlation was 96%.

13. LIMITATIONS OF USE

Specimen
Use serum only. The clotted samples must be centrifuged as soon as possible. Do not use hemolyzed or lipemic specimens. Only serum should be used for measuring PAPP-A, since Heparin, EDTA as well as other anticoagulants may affect the apparent concentration of PAPP-A of the sample.
Avoid multiple freeze-thaw cycles for any specimen.

Interfering substances
Heterophilic antibodies in human serum can react with the immunoglobulins included in the assay components causing interference with in vitro immunoassays. Samples from patients routinely exposed to animals or animal serum products can demonstrate this type of interference potentially causing an abnormal result.

Hook Effect
No hook effect was observed in this test up to 1785,7 mg/L of PAPP-A.

14. THERAPEUTICAL CONSEQUENCES

Therapeutical consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 9.1. Any laboratory result is only a part of the total clinical picture of a patient. Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutical consequences be derived. The test result itself should never be the sole determinant for deriving any therapeutical consequences.

15. COMPLAINTS

Complaints can be accepted in written format. All details of the test kit, as well as the test results, must be included.