**PAPP-A2 ELISA**

**INTENDED USE**
The PAPP-A2 enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of PAPP-A2 in human serum and other biological fluids.

**SUMMARY AND EXPLANATION**
Pregnancy associated plasma protein-A2 (PAPP-A2) is a metalloproteinase protein identified as a homolog of PAPP-A in the metzincin superfamily of pappalysins.\(^1\) PAPP-A2 is a noncovalently linked dimer of two 220-kDa subunits.\(^1\) PAPP-A2 cleaves insulin-like growth factor-binding protein 5 (IGFBP5) and Insulin like growth factor binding protein 3.\(^2\) PAPP-A2 is function as a local regulator of insulin-like growth factor (IGF) bioavailability.\(^3\) PAPP-A2 is expressed in a wide range of tissues and abundantly present in the placental syncytiotrophoblast and the pregnant uterus.\(^4\) The insulin-like growth factors are important in human embryo implantation and placenta.\(^5\) Numerous studies have identified the associations between the placental levels of PAPP-A2 and preeclampsia.\(^6\) It is being suggested that either altered levels PAPP-A2 cause abnormal placental development or the production of these proteins is altered to compensate for abnormal placenta.\(^7\)

**PRINCIPLE OF THE TEST**
The PAPP-A2 ELISA is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to anti-PAPP-A2 antibody coated micro titer wells and incubated. After first incubation and washing step, the wells are incubated with biotin labelled antibody conjugate. After a second incubation and washing step, the wells are incubated with streptavidin horseradish peroxidase conjugate (SHRP) solution. After the third incubation and washing step, the wells are incubated with substrate solution (TMB). After TMB incubation, an acidic stopping solution is added. In principle, the antibody-biotin conjugate binds to the solid phase antibody-antigen complex which in turn binds to the streptavidin enzyme conjugate. The antibody-antigen-biotin conjugate SHRP complex bound to the well is detected by enzyme-substrate reaction. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as reference filter. The absorbance measured is directly proportional to the concentration of PAPP-A2 in the samples and calibrators.

**MATERIALS SUPPLIED**

<table>
<thead>
<tr>
<th>CAL-109A</th>
<th>PAPP-A2 Calibrator A/Sample Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>One bottle, 10 mL, labeled PAPP-A2 Cal A/Sample Diluent, containing 0 ng/mL PAPP-A2 in a protein-based buffer with a non-mercury preservative. Store unopened at 2-8°C until the expiration date.</td>
<td></td>
</tr>
</tbody>
</table>

**CAL-109B - CAL-109F | PAPP-A2 Calibrators B thru F (Lyophilized)**

| Five vials, labeled B-F containing concentrations of approximately 0.09 – 9.4 ng/mL PAPP-A2 in protein-based buffer with non-mercury preservative. Refer to calibration card for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators B-F with 1.0 mL deionized water. Solubilize for 10 minutes, mix well and use after reconstitution. Discard after 5 days, if stored at 2 to 8°C. For longer storage after reconstitution, aliquot and freeze at -20°C or colder for up to one year. |

**MATERIALS REQUIRED BUT NOT SUPPLIED**

1. Microtitration plate reader capable of absorbance measurement at 450 nm, 405 nm and 630 nm.
2. Microtitration orbital plate shaker.
3. Microtitration plate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Vortex mixer.
6. Deionized water.
7. Disposable 12 x 75 mm culture tubes.
8. Tight fitting 12 x 75 mm tube racks.

**WARNINGS AND PRECAUTIONS**

*For in vitro diagnostic use.*

The following precautions should be observed:

a) Follow good laboratory practice.

b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunocassay materials.

c) Handle and dispose of all reagents and material in compliance with applicable regulations

**WARNING: Potential Biohazardous Material**

This reagent may contain some human source material (e.g. serum) or materials used in conjunction with human source materials. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition, 2007.

**WARNING: Potential Chemical Hazard**

Some reagents in this kit contain Pro-Clean 400 and Sodium azide as a preservative. Pro-Clean 400 and Sodium azide in concentrated amounts are irritants to skin and mucous membranes.

For further information regarding hazardous substances in the kit, please refer to the MSDS, either at AnshLabs.com or by request.

**SAMPLE COLLECTION AND PREPARATION**

a) Serum is the recommended sample type.

b) Use the following recommendations for handling, processing and storing blood samples:

- Allow samples to clot for two hours at room temperature or overnight at 4°C and follow blood collection tube manufacturer’s recommendations for centrifugation. Keep tubes stoppered always. Within two hours after centrifugation, transfer at least 500 µL of cell free sample to a storage tube. Tightly stopper the tube immediately.

- Samples if used within 24 hours may be stored at 4°C; otherwise samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.

- Remove residual fibrin and cellular matter prior to analysis.

c) Avoid assaying lipemic, hemolyzed or icteric samples.

d) Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products.

e) Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.

**PROCEDURAL NOTES**

1. A thorough understanding of this package insert is necessary for successful use of the PAPP-A2 ELISA assay. It is the responsibility of the customer to validate the assay for their use. Accurate results will only be obtained by using precise laboratory techniques and following the package insert.

2. A calibration curve must be included with each assay.

3. Bring all kit reagents to room temperature before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix different lots of any kit component and do not use any component beyond the expiration date.

4. Use a clean disposable pipette tip for each reagent, calibrator, control or sample. Avoid microbial contamination of reagents, contamination of the substrate solutions with the enzyme conjugates. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.

5. Incomplete washing will adversely affect the outcome and assay precision. Care should be taken to add TMB into the wells to minimize potential assay drift due to variation in the TMB incubation time. Avoid exposure of the reagents to excessive heat or direct sunlight.

**PREPARATION OF REAGENTS**

1. **PAPP-A2 Calibrators B-F and Controls I & II:** Tap and reconstitute PAPP-A2 Calibrators B-F and Controls I & II each with 1 mL deionized water. Solubilize for 10 minutes, mix well and use after reconstitution.

2. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature when stored in a tightly sealed bottle.

3. **Wash Solution as Sample Diluent:** Wash Concentrate A should be diluted at a ratio of 1 part into 24 parts of the deionized water, per the number of wells used. For an entire plate, pipet exactly 10 mL of the Wash Concentrate A into 240 mL of the deionized water, mix well and label as sample diluent.

4. **PAPP-A2 Antibody-Biotin Conjugate Solution:** The PAPP-A2 Antibody-Biotin Conjugate Concentrate should be diluted at a ratio of 1-part conjugate to 50 parts of PAPP-A2 Conjugate Diluent, according to the number of wells used. If an entire plate is to be used pipet exactly 220 µL of the Concentrate into 11 mL of the PAPP-A2 Conjugate Diluent.

5. **Microtiter Wells:** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

**Sample Preparations**

Dilution of pregnancy serum specimens should be performed on the same day of testing.

1. Prepare Sample Diluent as per the procedure detailed in the Preparation of Reagents section.

2. First trimester samples are diluted 1:20 and second trimester and till-term samples are diluted 1:80.

For First Trimester samples:

Dilution of serum specimens should be performed on the same day prior to testing and requires a 1:20 folds dilution.

- For each unknown serum sample, label one 12 X 75 culture tubes.
- Add 190 µL of the Sample Diluent to each tube.
- Add 10 µL of the serum specimens to the pre-labeled tube and vortex well.
- The sample is now ready for analysis.

For Second trimester and till-term samples (14 wks. or higher):

Dilution of serum specimens should be performed on the same day prior to testing and requires a 1:80 folds dilution.

- For each unknown serum sample, label one 12 X 75 culture tubes.
- Add 790 µL of the Sample Diluent to each tube.
- Add 10 µL of the serum specimens to the pre-labeled tube and vortex well.
- The sample is now ready for analysis.

**ASSAY PROCEDURE**

Allow all specimens and reagents to reach room temperature and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

**NOTE:** All diluted pregnancy samples (Refer sample preparations section) reading higher than the highest calibrator should be further diluted two-four folds in sample diluent prior to assay.

*Do not dilute Calibrators or Controls.*
1. Mark the microtitration strips to be used.
2. Pipette 50 µL of the Calibrator, Controls and Unknowns (neat for non-pregnancy and diluted for pregnancy serum specimens) to the appropriate wells.
3. Add 50 µL of the PAPP-A2 Assay Buffer to each well using a repeater pipette.
4. Incubate the wells, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 1 hour at room temperature (23 ± 2°C).
5. With 30-40 minutes remaining of incubation time, prepare the PAPP-A2 Antibody-Biotin Conjugate Solution by diluting the PAPP-A2 Biotin Conjugate Concentrate in PAPP-A2 Conjugate Diluent as described under the Preparation of the Reagents section of this insert.
6. Aspirate and wash each well 5 times with Wash Solution (350 µL per well) using an automatic microplate washer.
7. Add 100 µL of the Antibody-Biotin Conjugate solution to each well using a repeater pipette.
8. Incubate the wells, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 1 hour at room temperature (23 ± 2°C).
9. Aspirate and wash each well 5 times with the Wash Solution (350 µL per well) using an automatic microplate washer.
10. Add 100 µL of the Streptavidin-Enzyme Conjugate-RTU to each well using a repeater pipette.
11. Incubate the wells, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 30 minutes at room temperature (23 ± 2°C).
12. Aspirate and wash each well 5 times with the Wash Solution (350 µL per well) using an automatic microplate washer.
13. Add 100 µL of the TMB chromogen solution to each well using a repeater pipette. Avoid exposure to direct sunlight.
14. Incubate the wells, shaking at 600-800 rpm on an orbital microplate shaker, for 10-12 min at room temperature (23 ± 2°C).
   **NOTE:** Visually monitor the color development to optimize the incubation time.
15. Add 100 µL of the Stopping solution to each well using a repeater pipette. Read the absorbance of the solution in the wells within 20 minutes, using a microplate reader set to 450 nm.
   **NOTE:** Zero calibrator should be programmed as "Blank" while reading the optical density. If instrument has a wavelength correction, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction at 630 nm.

**RESULTS**

**NOTE:** The results in this package insert were calculated by plotting the log optical density (OD) data on the y-axis and log PAPP-A2 concentration on x-axis using a cubic regression curve-fit. Alternatively, log vs. log quadratic regression curve-fit can be used. Other data reduction methods may give slightly different results.

1. Optimum results can be obtained at incubation temperature of 23 ± 2°C.
2. Calculate the mean absorbance for each calibrator, Control, or Unknown. Plot the log of the mean absorbance readings for each of the Calibrators along the y-axis versus log of the PAPP-A2 concentrations in ng/mL along the x-axis, using a cubic regression curve-fit.
3. Determine the PAPP-A2 concentrations of the Controls and unknowns from the calibration curve by matching their mean absorbance readings with the corresponding PAPP-A2 concentrations.
4. Multiply the results with the dilution factor.
5. Any sample reading higher than the highest Calibrator should be appropriately diluted with the sample diluent and re-assayed. Multiply the value by a dilution factor.
6. Any sample reading lower than the analytical sensitivity should be reported as such.

**LIMITATIONS**

The reagents supplied in this kit are optimized to measure PAPP-A2 levels in human serum. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the samples.

**QUALITY CONTROL**

a) Each laboratory should establish mean values and acceptable ranges to assure proper performance.

b) PAPP-A2 ELISA controls or other commercial controls should fall within established confidence limits.

c) The confidence limits for PAPP-A2 ELISA controls are printed on the control vial labels.

d) A full calibration curve, low and high-level controls, should be included in each assay.

e) The TMB chromogen solution should be colorless. Development of a blue color may indicate reagent contamination or instability.

**REPRESENTATIVE CALIBRATION CURVE DATA**

<table>
<thead>
<tr>
<th>Well Number</th>
<th>Well Contents</th>
<th>Mean Absorbance</th>
<th>Conc.(ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1, A2</td>
<td>Calibrators</td>
<td>0.039 (Blank)</td>
<td>0</td>
</tr>
<tr>
<td>B1, B2</td>
<td>A</td>
<td>0.029</td>
<td>0.09</td>
</tr>
<tr>
<td>C1, C2</td>
<td>B</td>
<td>0.088</td>
<td>0.27</td>
</tr>
<tr>
<td>D1, D2</td>
<td>C</td>
<td>0.35</td>
<td>1.15</td>
</tr>
<tr>
<td>E1, E2</td>
<td>D</td>
<td>1.18</td>
<td>3.9</td>
</tr>
<tr>
<td>F1, F2</td>
<td>E</td>
<td>2.62</td>
<td>9.4</td>
</tr>
</tbody>
</table>

**CAUTION:** The above data must not be employed in lieu of data obtained by the user in the laboratory.

**ANALYTICAL CHARACTERISTICS**

All analytical characteristics are stated ng/mL.

Limit of Detection (LoD):
The lowest amount of PAPP-A2 in a sample that can be detected with a 95% probability (n=24) is 0.071 ng/mL. The value was determined by processing a complete six-point calibration curve, controls and six serum samples in the range of 0.091 to 3.085 ng/mL following CLSI EP17 guidelines. Three assay runs per day were performed over four days with all samples run in duplicate per run.

Limit of Quantitation (LoQ):
The estimated minimum PAPP-A2 dose achieved at 20% total imprecision is 0.08 ng/mL. The value was determined by processing a complete six-point calibration curve, controls and eight samples in the range of 0.091-3.08 ng/mL over twelve runs and four days in duplicates (n=24) following CLSI EP17 guidelines.

Reproducibility of the PAPP-A2 ELISA assay was determined in a study using four serum pools. The study included a total of 12 assays, four replicates of each per assay (n=48). Representative data were calculated based on NCCLS EP5-A guidelines and are presented in the following table.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean conc. Within run</th>
<th>Between run</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ng/mL)</td>
<td>SD</td>
<td>%CV</td>
</tr>
<tr>
<td>Pool-1</td>
<td>1.029</td>
<td>0.044</td>
<td>4.25%</td>
</tr>
<tr>
<td>Pool-2</td>
<td>1.813</td>
<td>0.059</td>
<td>3.28%</td>
</tr>
<tr>
<td>Pool-3</td>
<td>2.651</td>
<td>0.111</td>
<td>4.18%</td>
</tr>
<tr>
<td>Pool-4</td>
<td>3.128</td>
<td>0.115</td>
<td>3.68%</td>
</tr>
</tbody>
</table>

**Linearity:**
Based on NCCLS EP-6-P multiple dilutions of the four serum samples containing various PAPP-A2 levels were diluted with Calibrator A/sample.
diluent. The % recovery on individual samples is represented in the following table.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution Factor</th>
<th>Expected Conc. (ng/mL)</th>
<th>Observed Conc. (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:10</td>
<td>8.134</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>4.067</td>
<td>4.239</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>2.034</td>
<td>2.126</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>1:80</td>
<td>1.017</td>
<td>1.070</td>
<td>105</td>
</tr>
<tr>
<td>2</td>
<td>1:10</td>
<td>7.541</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>3.771</td>
<td>3.808</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>1.885</td>
<td>1.996</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>1:80</td>
<td>0.943</td>
<td>1.025</td>
<td>109</td>
</tr>
<tr>
<td>3</td>
<td>1:10</td>
<td>6.636</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>3.318</td>
<td>3.599</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>1.659</td>
<td>1.751</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>1:80</td>
<td>0.830</td>
<td>0.882</td>
<td>106</td>
</tr>
<tr>
<td>4</td>
<td>1:10</td>
<td>5.245</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>2.623</td>
<td>2.693</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>1.311</td>
<td>1.372</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>1:80</td>
<td>0.656</td>
<td>0.824</td>
<td>126</td>
</tr>
</tbody>
</table>

Recovery:
Known amounts of PAPP-A2 were added to four serum samples containing different levels of endogenous PAPP-A2. The concentration of PAPP-A2 was determined before and after the addition of exogenous PAPP-A2 and the percent recovery was calculated.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous Conc.(ng/mL)</th>
<th>Expected Conc. (ng/mL)</th>
<th>Observed Conc. (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.297</td>
<td>0.788</td>
<td>0.675</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>1.179</td>
<td>0.978</td>
<td>0.978</td>
<td>83</td>
</tr>
<tr>
<td>2</td>
<td>0.302</td>
<td>0.793</td>
<td>0.699</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>1.184</td>
<td>1.058</td>
<td>1.058</td>
<td>89</td>
</tr>
<tr>
<td>3</td>
<td>0.329</td>
<td>0.819</td>
<td>0.698</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>1.208</td>
<td>1.009</td>
<td>1.009</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>0.53</td>
<td>1.009</td>
<td>0.934</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>1.39</td>
<td>1.167</td>
<td>1.167</td>
<td>84</td>
</tr>
</tbody>
</table>

Analytical Specificity:

Interference:
When potential interferences (hemoglobin, triglycerides and bilirubin) were added at least at two times their physiological concentration to control sample, PAPP-A2 concentration were within ± 15% of the control as represented in the following table. This study was based on NCCLS EP7-P to serum matrix added

<table>
<thead>
<tr>
<th>Interferents</th>
<th>Analyte Conc. (mg/mL)</th>
<th>Non-spiked Sample Value (ng/mL)</th>
<th>Spiked Sample Value (ng/mL)</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>1.35</td>
<td>0.221</td>
<td>0.229</td>
<td>3.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.271</td>
<td>0.292</td>
<td>7.75</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>11</td>
<td>0.221</td>
<td>0.213</td>
<td>-3.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.358</td>
<td>0.343</td>
<td>-4.19</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.6</td>
<td>0.214</td>
<td>0.225</td>
<td>5.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.271</td>
<td>0.286</td>
<td>5.53</td>
</tr>
</tbody>
</table>

Expected Value:
These samples were analyzed using PAPP-A2 ELISA kit on site. The expected ranges for PAPP-A2 were calculated using 90-95% non-parametric estimation using Analyse-it® for Microsoft Excel.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Median Conc. (ng/mL)</th>
<th>2.5–97.5th Percentile Conc. (ng/mL)</th>
</tr>
</thead>
</table>

Note: The values above are for reference only. It is recommended that each research organization should determine the reference values for their own study population.

Method Comparison:
A total of 178 pregnancy (18-34 wks.) serum samples from 110 controls and 68 preeclampsia subjects were analyzed. The ROC analysis showed an area under the curve of 0.99 and the frequency distributions of the two groups with respect to PAPP-A2 concentrations are shown in the figure below.

**REFERENCES**
5. Winn VD et al. Severe preeclampsia-related changes in gene expression at the maternal-fetal interface include sialic acid-binding immunoglobulin-like lectin-6 and pappalysin-2. Endocrinology. 2009; 150:452-62

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