

# PAPP- A2 ELISA

## AL-109

# RUO

### 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. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

### SUMMARY AND EXPLANATION

Pregnancy associated plasma protein-A2 (PAPP-A2) is a novel metalloproteinase identified as a homolog of PAPP-A in the metzincin superfamily of pappalysins<sup>1-8</sup>. PAPP-A2 shares 46% sequence identity with PAPP-A<sup>3</sup>. PAPP-A2 is a noncovalently linked dimer of two 220-kDa subunits. It exhibits robust proteolytic activity against IGFBP-5 and possibly also IGFBP-3<sup>3, 8</sup>. PAPP-A2 is expressed in a wide range of tissues and is abundant in placental syncytiotrophoblasts and the pregnant uterus<sup>3</sup>. The physiological importance of PAPP-A2 is not known.

### 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 step, 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

#### CAL-109A PAPP-A2 Calibrator A/Sample Diluent

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.

#### CAL-109B -CAL-109F PAPP-A2 Calibrators B thru F

Five vials, labeled B-F containing a concentration 0.1, 0.3, 1.2, 4.0 and 10 ng/mL PAPP-A2 in protein based buffer with non-mercury preservative. Calibrators are shipped ambient. **Store at - 20°C upon receipt** until the expiration date. The PAPP-A2 concentration in the PAPP-A2 calibrators is traceable to manufacturer's working calibrators. Values assigned by other methodologies may be different. Such differences, if present, may be caused by inter-method bias.

#### CTR-109-I and CTR-109-II PAPP-A2 Controls I & II

Two vials, labeled Levels I and II containing low and high PAPP-A2 in protein based buffer with non-mercury preservative. Controls are shipped ambient. **Store at - 20°C upon receipt** until the expiration date.

#### PLT-109 Anti-PAPP-A2 Antibody Coated Microtitration Strips

One strip holder, containing 96 microtitration wells with PAPP-A2 antibody immobilized to the inside wall of each well. Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

#### ASB-109 PAPP-A2 Assay Buffer

One bottle, 8 mL, containing a protein-based (BSA)-buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

#### CND-109 PAPP-A2 Biotin Conjugate Diluent

One bottle, 12 mL, containing a protein based buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

#### BCC-109 PAPP-A2 Biotin Conjugate Concentrate

One vial, 0.4 mL containing a solution of anti-PAPP-A2 antibody biotin concentrate in a protein-based buffer with a non-mercury preservative. Dilute prior to use in PAPP-A2 Conjugate diluent. Store at 2-8°C until expiration date.

**NOTE:** The dilution of this reagent should be made 15-30 minutes prior to use in the assay.

#### SAR-109 PAPP-A2 Streptavidin-Enzyme Conjugate—Ready-to-Use (RTU)

One amber bottle, 12 mL, containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative. Store undiluted at 2-8°C until expiration date.

#### TMB-100 TMB Chromogen Solution

One bottle, 11 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2-8°C until expiration date.

#### STP-100 Stopping Solution

One bottle, 11 mL, containing 0.2 M sulfuric acid. Store at 2 to 30°C until expiration date.

#### WSH-100 Wash Concentrate A

One bottle, 60 mL, containing buffered saline with a nonionic detergent. Store at 2-30°C until expiration date. Dilute 25-fold with deionized water prior to use.

### MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microtitration plate reader capable of absorbance measurement at 450 nm, 405nm 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 Research Use Only. Not for use in diagnostic procedures.**

The following precautions should be observed:

- a) Follow good laboratory practice.

- b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay 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," 5<sup>th</sup> Edition, 2007<sup>9</sup>.

#### **WARNING: Potential Chemical Hazard**

Some reagents in this kit contain Pro-Clean 400 and Sodium azide<sup>10</sup> 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.<sup>11</sup>
  - 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 at all times. 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 accurately and efficiently to minimize potential assay drift due to variation in the TMB incubation time. Avoid exposure of the reagents to excessive heat or direct sunlight.

#### **TEST PROCEDURE**

##### **Preparation of Reagents**

1. **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.
2. **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 in to 11 mL of the PAPP-A2 Conjugate Diluent.
3. **Microtitration 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.

#### **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 serum samples reading higher than the highest calibrator should be thoroughly mixed and diluted in the 0 ng/mL PAPP-A2 Cal A/Sample diluent prior to assay.

*For pregnancy serum specimens: Dilute specimens 1:20 with the Cal A/Sample diluent before 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 to the appropriate wells.
3. Add **50 µL** of the PAPP-A2 Assay Buffer to each well using a semi-automatic dispenser.
4. Incubate the wells, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for **1 hour** at room temperature.
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** for 30 seconds with Washing Solution using an automatic microplate washer.
7. Add **100 µL** of the Antibody-Biotin Conjugate solution to each well using a semi-automatic dispenser.
8. Incubate the wells, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for **1 hour** at room temperature.
9. Aspirate and wash each well 5 times with the Wash Solution using an automatic microplate washer.
10. Add **100 µL** of the Streptavidin-Enzyme Conjugate-RTU to each well using a semi-automatic dispenser.
11. Incubate the wells, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for **30 minutes** at room temperature.
12. Aspirate and wash each well **5 times** with the Wash Solution using an automatic microplate washer.
13. Add **100 µL** of the TMB chromogen solution to each well using a precision 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.  
**NOTE: Visually monitor the color development to optimize the incubation time.**
15. Add **100 µL** of the stopping solution to each well using a precision pipette.
16. Read the absorbance of the solution in the wells within 20 minutes, using a microplate reader set to 450 nm.

**NOTE:** While reading the absorbance of the microtitration well, it is necessary to program the zero calibrator as a "Blank".

## RESULTS

**NOTE:** The results in this insert were calculated by plotting the data on a log vs. log scale using a cubic regression curve-fit. Other data reduction methods may give slightly different results.

1. 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.
2. 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.
3. Any sample reading higher than the highest Calibrator should be appropriately diluted with the 0 ng/mL (CAL A/Sample diluent) and re-assayed. For pregnancy samples: Dilute 1:20 with the 0 ng/mL (Cal A/Sample diluent) before assay. Multiply the value by a dilution factor.
4. 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<sup>12</sup>.

## 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

Well Number	Well Contents	Mean Absorbance	Conc (ng/mL)
<b>Calibrators</b>			
A1, A2	A	0.060 (Blank)	0
B1, B2	B	0.038	0.1
C1, C2	C	0.110	0.3
D1, D2	D	0.431	1.2
E1, E2	E	1.433	4.0
F1, F2	F	3.079	10.0

**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.

### Imprecision:

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.

Sample	Mean conc. (ng/mL)	Within run		Between run		Total	
		SD	%CV	SD	%CV	SD	%CV
Pool-1	1.029	0.044	4.25%	0.020	1.94%	0.048	4.67%
Pool-2	1.813	0.059	3.28%	0.046	2.53%	0.075	4.14%
Pool-3	2.651	0.111	4.18%	0.050	1.89%	0.122	4.59%
Pool-4	3.128	0.115	3.68%	0.076	2.42%	0.138	4.41%

### 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.

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

### 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.

Sample	Endogenous Conc.(ng/mL)	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1	0.297	0.788	0.675	86
		1.179	0.978	83
2	0.302	0.793	0.699	88
		1.184	1.058	89
3	0.329	0.819	0.698	85
		1.208	1.009	84
4	0.53	1.009	0.934	93
		1.39	1.167	84

**Analytical Specificity:**

The antibody pair used in the PAPP-A2 ELISA measures PAPP-A2 and does not detect other similar molecules. The assay does not detect dimeric PAPP-A, PAPP-A proMBP complex, proMBP and MMP-9.

**Interference:**

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

Interferents	Analyte Conc. (mg/mL)	Unspiked Sample Value (ng/mL)	Spiked Sample Value (ng/mL)	% Difference
Hemoglobin	1.35	0.221	0.229	3.62
		0.271	0.292	7.75
Triglycerides	11	0.221	0.213	-3.62
		0.358	0.343	-4.19
Bilirubin	4.2	0.214	0.225	5.14
		0.271	0.286	5.53

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