Monobind Inc.
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AccuLite
CLIA Microwells

Pregnancy Associated Plasma Protein-A (PAPP-A) Test System

Product Code: 12675-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of PAPP-A Concentration in Human Serum by a Microplate Enzyme Immunoassay, Chemiluminescence

2.0 SUMMARY AND EXPLANATION OF THE TEST

Trisomy 21, 18, and 13 are three conditions that can cause ill effects to a fetus and, therefore, an infant. Screening for markers and inherent trends has been of interest over the years. One of the proteins of interest for these trisomy disorders is PAPP-A, pregnancy associated plasma protein A. This protein can be found in the serum of every individual, but the concentration rises in maternal serum as gestation time progresses. In studies, a correlation has been shown between the decreased level of PAPP-A and the occurrence of trisomy disorders, particularly Trisomy 21, also known as Down's Syndrome. Along with a few other markers like AFP, uE3, and hCG, trends in PAPP-A have been found to elude to trisomy disorders.[1,2,3,4,5,6]

PAPP-A is produced primarily by the placenta during pregnancy. This glycoprotein has a molecular weight of 740,000 and tends to exist as a heterotetrameric dimer with ProMBP, proform major basic protein. The concentration of PAPP-A in maternal blood increases over the time of the pregnancy as the placenta and fetus grows because it is a product of the trophoblast. In general, the concentration level of this protein in maternal serum is indicative of threatened abortion, preterm birth, intrauterine growth restriction, ectopic gravidity, preeclampsia or diabetes mellitus. When tested during the first trimester of pregnancy, PAPP-A is the major marker for Down's syndrome.[3,7,8,9]

The Monobind PAPP-A test system is designed specifically for the testing of the heterotetrameric form important during pregnancy. Another form of PAPP-A also exists in serum, but it is a dimeric form associated with coronary and cardiac conditions. Tests developed for use on pregnancy patients are not designed to test for this dimeric form. When evaluation of this protein is done, it is often compared to the Multiple of Medians (MoM) and represented as a percent of an in-house establish median. Without easy access to an IRP for PAPP-A, this method allows an easier way to compare results between laboratories and testing methods, allowing for a type of reference value to be established.[2,5,8,9]

3.0 PRINCIPLE

Immunoenzymometric sequential assay (Type 4):
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, **in excess,** and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-PAPP-A antibody.

Upon mixing monoclonal biotinylated antibody, and a serum containing the native antigen, reaction results between the native antigen and the antibody, forming an Antibody-Antigen complex. The interaction is illustrated by the following equation**:**

$$Ag_{(PAPP\text{-}A)} + {}^{Btn}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} Ag_{(PAPP\text{-}A)} - {}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)
$Ag_{I(PAPP\text{-}A)}$ = Native Antigen (Variable Quantity)
$Ag_{(PAPP\text{-}A)}$ - $^{Btn}Ab_{(m)}$= Antigen-Antibody complex (Variable Quan.))
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:
$Ag_{(gE)}$ - $^{Btn}Ab_{(m)}$ + $\underline{\text{Streptavidin}}_{C.W.} \Rightarrow \underline{\text{Immobilized complex}}$ (IC)
$\underline{\text{Streptavidin}}_{C.W.}$ = Streptavidin immobilized on well
$\underline{\text{Immobilized complex (IC)}}$ = Ag-Ab bound to the well

After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation or aspiration. Another antibody (directed at a different epitope) labeled with an enzyme is added. Another interaction occurs to form an enzyme labeled antibody-antigen-biotinylated-antibody complex on the surface of the wells. Excess enzyme is washed off via a wash step. A suitable substrate is added to produce light measurable with the use of a microplate luminometer. The enzyme activity on the well is directly proportional to the native free antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

$$(IC) + {}^{Enz}Ab_{(x\text{-}PAPP\text{-}A)} \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} {}^{Enz}Ab_{(x\text{-}PAPP\text{-}A)} - IC$$

$^{Enz}Ab_{(x\text{-}PAPP\text{-}A)}$ = Enzyme labeled Antibody (Excess Quantity)
$^{Enz}Ab_{(x\text{-}PAPP\text{-}A)}$ – IC = Antigen-Antibodies Complex
k_b = Rate Constant of Association
k_{-b} = Rate Constant of Dissociation

4.0 REAGENTS

Materials Provided:

A. PAPP-A Calibrators – 0.5 ml/vial – Icons A-F
Six (6) vials of serum reference for PAPP-A containing concentrations of 0 (**A**), 0.64 (**B**), 1.6 (**C**), 3.2 (**D**), 9.6 (**E**) and 32 (**F**) in µg/ml. A preservative has been added. Store at 2-8°C.
Note: To convert µg/ml to mIU/L, multiply by 156.25
Ex. 32 µg/ml x 156.25 = 5000 mIU/L

B. PAPP-A Tracer Reagent– 12 ml/vial-Icon E
One (1) vial contains PAPP-A (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix. Store at 2-8°C.

C. PAPP-A Biotin Reagent – 12 ml/vial – Icon ∇
One (1) vial containing biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.
Note: Patients must be fasting from biotin therapy/supplements for 8 hours to prevent interference in the test

D. PAPP-A Control – 0.5 ml/vial – Icon M
One (1) vials of serum reference for PAPP-A at concentration established (exact value listed on label). A preservative has been added. Store at 2-8°C.

E. PAPP-A Diluent – 5.0 ml/vial – Icon U
One (1) vial of human serum based buffer with salts, surfactants, and preservatives. Store at 2-8°C.

F. Streptavidin Reaction Wells 96 wells – Icon ⇓
One 96-well white microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

G. Wash Solution Concentrate – 20ml/vial – Icon
One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

H. Signal Reagent A – 7 ml/vial – Icon C^A
One (1) vial contains luminol in buffer. Store at 2-8°C (see Reagent Preparation section).

I. Signal Reagent B – 7 ml/vial – Icon C^B
One (1) vial contains hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C (see Reagent Preparation section).

J. Product Instructions.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. **Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on label.**
Note 3: Above reagents are for a single 96-well microplate**.**

Required But Not Provided:
1. Pipette capable of delivering 0.010ml (10µl), 0.050ml (50µl) and 0.100ml (100µl) with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.
3. Microplate washer or a squeeze bottle (optional).
4. Microplate Luminometer
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate covers for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Quality control materials.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum in type, and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants; a red top gel separator tube (also known as a "Tiger" top tube) can be used. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.020ml of the specimen is required.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and high range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. **Wash Buffer**
Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.
2. **Working Signal Reagent Solution –** Store at 2 - 8°C.
Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).
*****Test procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.010ml (10µL) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100ml (100µl) of the PAPP-A Biotin Reagent to all wells.
4. Swirl the microplate gently for 20-30 seconds to mix.
5. Cover and incubate for 30 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
7. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.**
8. Add 0.100ml (100µl) of PAPP-A Tracer Reagent to all wells.
DO NOT SHAKE (MIX) THE PLATE AFTER TRACER ADDITION
9. Cover and incubate for 30 minutes at room temperature
10. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
11. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate.
12. Repeat four (4) additional times for a total of five (5) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.**
13. Add 0.100ml (100µl) of working Signal Reagent to all wells. **Always add reagents in the same order to minimize reaction time differences between wells.**
DO NOT SHAKE (MIX) THE PLATE AFTER SIGNAL ADDITION
14. Incubate for five (5) minutes at room temperature in the dark.
15. Read the relative light units (RLUs) in each well for 0.2 – 1.0 seconds. **The results should be read within thirty (30) minutes of adding the signal reagent solution.**

Note 1: **Do not use the working signal reagent solution if older than 36 hours.**
Note 2: **Do not use reagents that are contaminated or have bacteria growth.**
Note 3: **Cycle (start and stop) mixing (4 cycles) for 5-8 seconds/cycle is more efficient than one continuous (20-30 seconds) cycle to achieve homogeneity. A plate mixer can be used to perform the mixing cycles.**
Note 4: **It is extremely important to accurately dispense the correct volume with a calibrated pipette and by**

adding near the bottom of the microwells at an angle while touching the side of the well.

Note: Dilute the samples suspected of concentrations higher than 32 µg/ml with PAPP-A diluent and multiply result by dilution factor. For a 1:5 dilution add 40µl of diluent 10µl of high concentration sample.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of PAPP-A in unknown specimens.

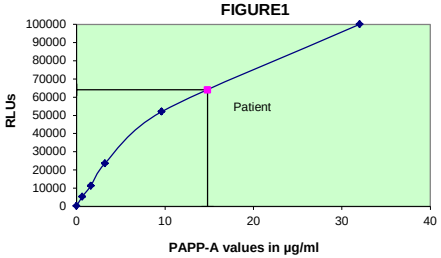
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding PAPP-A concentration in µg/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Connect the points with a best-fit curve.
4. To determine the concentration of PAPP-A for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance in the patient/ sample (526341) intersects the dose response curve at (10.5 µg/ml) PAPP-A concentration (See Figure 1).

Note: Computer data reduction software designed for CLIA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Number	RLUs	Mean RLU (B)	Value (µg/ml)
Cal A	A1	206	223	0
	A2	240		
Cal B	B1	5517	5342	0.64
	B2	5167		
Cal C	A6	10425	11300	1.6
	B6	12175		
Cal D	C6	24329	23598	3.2
	D1	22866		
Cal E	E1	51308	51967	9.6
	E2	52626		
Cal F	F1	103380	100000	32.0
	F2	96620		
Ctrl # 1	C3	30070	31118	4.8
	D3	32165		
Ctrl #2	E3	69117	71366	19.1
	F3	73615		
Patient	G1	65193	64036	14.8
	H1	62878		

FIGURE1



* The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay. In addition, the RLUs of the calibrators have been normalized to 100,000 RLUs for the F calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The dose response curve should be within the established parameters.
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product are available on request from Monobind Inc.

12.1 Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of signal reagent solution initiates a kinetic reaction; therefore, the solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.
8. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.
9. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
10. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
11. Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation

1. ***Measurements and interpretation of results must be performed by a skilled individual or trained professional.***
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for AccuLite® CLIA procedures have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of Immunoassays (Boscato LM, Stuart MC ''Heterophilic antibodies: a problem for all immunoassays'' *Clin. Chem* (1988:3427-33). For diagnostic purposes, the results from this assay should be in combination with clinical examination, patient history, and all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
6. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

13.0 EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal" adult population, the expected ranges for the PAPP-A AccuLite® CLIA Test System are detailed in Table 1.

It is recommended to compare values based on the Multiple of Median (MoM) established for the laboratory when assessing patient samples. By dividing the value of the patient sample by the MoM will give a percent value that is used frequently for evaluation.

TABLE I
Expected Values for the PAPP-A Test System

Term of Gestation (full weeks)	PAPP-A Concentration (µg/ml)
9	3.86
10	7.1
11	10.1
12	16.68
13	23.2

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The total precision of the PAPP-A AccuLite® CLIA Test System was determined by analyses on six different levels of pool control and patient sera. The mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 2.

TABLE 2
Precision data for the PAPP-A Test System

Sample	Mean Value (µg/ml)	Within-Run Precision		Total Precision (n=80)	
		SD	CV%	SD	CV%
Control 1	0.66	0.04	6.03	0.07	9.96
Control 2	2.14	0.12	5.41	0.18	8.48
Control 3	8.49	0.52	6.12	0.83	9.83
Patient 1	1.26	0.10	7.91	0.13	9.92
Patient 2	5.06	0.26	5.08	0.50	9.90
Patient 3	28.25	1.70	6.01	2.75	9.73

*As measured in forty experiments in duplicate over a 20 day period.

14.2 Sensitivity

The LoB/LoD/LoQ of the PAPP-A AccuLite® CLIA Test System was calculated in conformance with CLSI EP17-A2 Protocols for Determination of Limits of Detection of Limits of Quantitation. The LoB is 0.011 µg/ml and the LoD=LoQ=0.071 µg/ml.

14.3 Accuracy

14.3.1 Linearity

The linearity of the PAPP-A AccuLite® CLIA test system was tested by diluting human serum samples containing high levels of PAPP-A (10 to 37 µg/ml) with low PAPP-A diluent. The system produces excellent linearity through the range of the test up to 37 µg/ml.

14.3.2 Recovery

The recovery of the PAPP-A AccuLite® CLIA Test System was calculated for five patient samples spiked with 0.5, 2.0, 4.0, 10.0, and 30.0 µg/ml PAPP-A. Recoveries were determined to be within 15% of the expected values for all samples.

14.3.3 Method Comparison

The PAPP-A AccuLite® CLIA Test System was compared with a reference immunoassay method. Biological specimens from low, normal and relatively high PAPP-A level populations were used; the values ranged from 0.1 µg/ml – 36 µg/ml. The total number of such specimens was 50. The least square regression equation and the correlation coefficient were computed for this PAPP-A ELISA in comparison with the reference method.

TABLE 4

Method	Mean	Least Square Regression Analysis	Correlation Coefficient
Monobind (y)	6.52	y = 0.34 + 0.97(x)	0.992
Reference (x)	6.68		

14.4 Specificity

The % cross-reactivity of the PAPP-A antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between doses of interfering substance to dose of PAPP-A needed to displace the same amount of labeled analog.

TABLE 5

Substance	Cross Reactivity
Free βhCG	ND
AFP	ND
hPRL	ND
FSH	ND

15.0 REFERENCES

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MP12675 **Product Code: 12675-300**

Size		96(A)	192(B)
Reagent (fill)	A)	0.5 ml set	0.5 ml set
	B)	1 (12ml)	2 (12 ml)
	C)	1 (12ml)	2 (12 ml)
	D)	0.5 ml set	0.5 ml set
	E)	1 (5.0 ml)	2 (5.0 ml)
	F)	1 plate	2 plates
	G)	1 (20ml)	1 (20ml)
	H)	1 (7ml)	2 (7ml)
	I)	1 (7ml)	2 (7ml)

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Glossary of Symbols
(EN 980/ISO 15223)