Quantikine® ELISA

Human Pappalysin-1/PAPP-A Immunoassay

Catalog Number DPPA00

For the quantitative determination of human Pregnancy-Associated Plasma Protein-A (PAPP-A) concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Pregnancy-Associated Plasma Protein-A (PAPP-A), also known as Pappalysin-1, is a 400 kDa secreted, homodimeric glycoprotein that belongs to the metzincin superfamily of metalloproteases (1, 2). It is best known as a regulator of IGF-II activity and plays a crucial role in placental development (1, 3). It is produced by a variety of primary cell types, including osteoblasts (4), vascular smooth muscle cells (5), fibroblasts (6), and follicular granulosa cells (7). The cells most associated with PAPP-A expression are endometrial stromal fibroblasts and extravillous trophoblasts (8-11). During placental growth, IGF-II seems to be the dominant insulin-like growth factor. It promotes steroidogenesis, glucose transport, and trophoblast (fetal tissue) invasion of the uterine endometrium (decidua). IGF-II is expressed by extra-embryonic mesoderm and extravillous cytotrophoblasts (non-fetal cells that anchor the developing embryo to the uterine wall). Upon secretion, IGF-II activity is quickly downregulated through its binding to IGFBP-4 produced by adjacent decidual fibroblasts (9, 10, 12). Activity is restored when IGF-II is released from IGFBP-4, a process that involves IGFBP-4 cleavage. PAPP-A is the enzyme responsible for this cleavage (13). It requires the presence of an IGF-II:IGFBP-4 complex and performs this action while non-covalently immobilized (presumably) on the surface of trophoblast cells (9, 11). PAPP-A itself is negatively regulated by a membrane-associated formation of a pro-MBP (major basic protein):PAPP-A heterotetramer (2:2 ratio) (14, 15). Pro-MBP is a 50-90 kDa trophoblast-derived protein that forms multiple disulfide bonds with PAPP-A and induces its dissociation from the trophoblast cell membrane. This creates an inactive, circulating, 500 kDa PAPP-A heteromeric complex (1). Not all circulating PAPP-A is part of an inactive heterotetramer. Approximately 1% of PAPP-A in pregnancy sera exists as a proteolytically active 400 kDa homodimer and significant quantities of homodimer have been noted in acute coronary syndrome (1, 3, 11, 16).

Human PAPP-A is synthesized as a 1628 amino acid (aa) prepro-precursor that contains a 22 aa signal sequence, a 58 aa pro-segment, and a 1548 aa mature region (17-19). The mature region contains an N-terminal laminin G-like module that stabilizes an adjacent proteolytic domain. The proteolytic domain (aa 324-672) shows a histidine-rich zinc-binding motif plus two LNR repeats that participate in IGFBP-4 cleavage. Five CCP/SCR class III modules (aa 1215-1554) exist at the C-terminus. Upon secretion, SCR-3 and -4 are believed to anchor PAPP-A to the trophoblast cell surface by binding to cell membrane heparan sulfate polymers (1, 11, 20). The PAPP-A 400 kDa homodimer consists of two parallel monomers linked by one disulfide bond (Cys1130-Cys1130). When pro-MBP combines with PAPP-A to generate an inhibitory 2:2 heterotetrameric complex, each pro-MBP monomer forms two disulfide bonds with its counterpart monomer and two disulfide bonds with one of the two PAPP-A monomers ($Cys381_{PAPP\text{-}A}$-$Cys51_{MBP}$ and $Cys652_{PAPP\text{-}A}$-$Cys169_{MBP}$) (21, 22). Over the mature region, human PAPP-A shows 91% aa sequence identity with mouse PAPP-A. There is one potential GenBank isoform of human PAPP-A that is 1232 aa in length. It shows an alternate start site at Met209 and a 22 aa substitution for the C-terminal 210 aa of the long form (23).

The Quantikine® Human Pappalysin-1/PAPP-A Immunoassay is a 5.5 hour sandwich-type solid phase ELISA designed to measure human PAPP-A in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human PAPP-A and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human PAPP-A showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural human PAPP-A.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human PAPP-A has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any PAPP-A present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human PAPP-A is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of PAPP-A bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- Samples must be pipetted within 15 minutes.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human PAPP-A Microplate	893567	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human PAPP-A.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human PAPP-A Conjugate	893568	21 mL of a polyclonal antibody specific for human PAPP-A conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human PAPP-A Standard	893569	Recombinant human PAPP-A in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-15	895424	11 mL of a buffered protein base with preservative.	
Calibrator Diluent RD6-14	895220	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- **Polypropylene** test tubes for dilution of standards.
- Human PAPP-A Controls (optional; R&D Systems®, Catalog # QC23).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

REAGENT PREPARATION

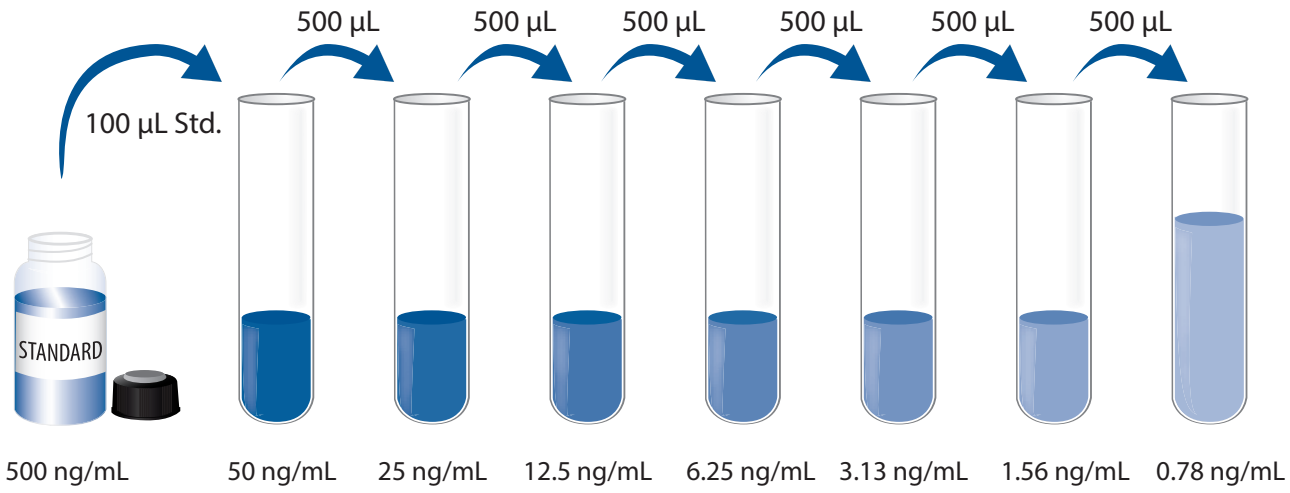
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Human PAPP-A Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human PAPP-A Standard with deionized or distilled water. This reconstitution produces a stock solution of 500 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD6-14 into the 50 ng/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 50 ng/mL standard serves as the high standard. Calibrator Diluent RD6-14 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

Note: *Strict adherence of the incubation times is critical to the performance of the assay.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-15 to each well.
4. Add 50 µL of standard, control, or sample per well. **Note:** *Pipetting must be completed within 15 minutes.* Cover with the adhesive strip provided. Incubate for **3 hours** at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human PAPP-A Conjugate to each well. Cover with a new adhesive strip. Incubate for **2 hours** at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for **30 minutes** at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

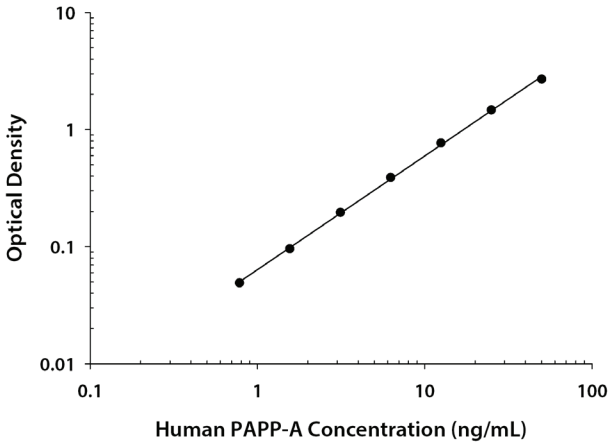
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human PAPP-A concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.011 0.011	0.011	—
0.78	0.059 0.060	0.060	0.049
1.56	0.105 0.108	0.107	0.096
3.13	0.199 0.214	0.207	0.196
6.25	0.391 0.411	0.401	0.390
12.5	0.763 0.795	0.779	0.768
25	1.448 1.514	1.481	1.470
50	2.682 2.729	2.706	2.695

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	5.49	17.4	32.7	5.20	16.4	31.4
Standard deviation	0.32	0.78	1.39	0.44	1.14	2.04
CV (%)	5.8	4.5	4.3	8.5	7.0	6.5

RECOVERY

The recovery of human PAPP-A spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	110	90-117%
Serum (n=4)	92	84-104%
EDTA plasma (n=4)	97	89-108%
Heparin plasma (n=4)	95	86-111%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human PAPP-A were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=8)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	102	104	105	100
	Range (%)	101-104	99-111	99-112	97-106
1:4	Average % of Expected	100	108	105	108
	Range (%)	97-103	100-114	103-114	105-114
1:8	Average % of Expected	97	107	107	111
	Range (%)	96-98	93-117	101-114	109-115
1:16	Average % of Expected	93	102	107	111
	Range (%)	90-96	92-110	95-115	106-116

SENSITIVITY

Fifty assays were evaluated and the minimum detectable dose (MDD) of human PAPP-A ranged from 0.015-0.116 ng/mL. The mean MDD was 0.053 ng/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human PAPP-A produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma:
Thirty-six serum and plasma samples from non-pregnant donors were evaluated for the presence of human PAPP-A in this assay. No detectable levels were observed. No medical histories were available for the donors used in this study.

Thirty pregnancy sera ranging from 6-40 weeks gestation were tested for human PAPP-A. Sample values ranged from non-detectable to 325 ng/mL.

Cell Culture Supernates - Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernate were removed and assayed for human PAPP-A. No detectable levels were observed.

SPECIFICITY

This assay recognizes natural and recombinant human PAPP-A.

The factors listed below were prepared at 500 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 500 ng/mL in a mid-range recombinant human PAPP-A control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

IGFBP-4
IGFBP-5
MBP-1
PAPP-A2
Serpin A8

Natural protein:

human α_2-macroglobulin

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