Quantikine® ELISA

Human α1-Acid Glycoprotein Immunoassay

Catalog Number DAGP00

For the quantitative determination of human α1-Acid Glycoprotein (AGP) concentrations in cell culture supernates, serum, plasma, and urine.

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

Human α1-acid Glycoprotein (AGP), also known as orosomucoid, is an acute-phase serum protein that is produced by the liver in response to inflammation and infection. It is a single polypeptide chain of 183 amino acids with a 42 kDa molecular weight. AGP is heavily glycosylated. It contains five to six highly sialylated complex-type-N-linked glycans, which represent about 45% of its molecular weight (1, 2). Although some extrahepatic expression has been reported, AGP is predominantly produced in the liver (3). AGP is a member of the lipocalin family.

Like most acute phase proteins, AGP synthesis in the liver increases in response to various systemic tissue injuries, such as inflammation and infection. Its serum concentration can increase 2-5 fold, making it one of the predominant proteins in serum (1). Expression of the AGP gene is regulated by a number of inflammation modulators, such as glucocorticoids and cytokines (4). Both *in vitro* and *in vivo* studies have shown that IL-6 and IL-1 are strong inducers for AGP. Besides being an acute phase reactant, AGP also has a number of other physiological functions. It can function as a carrier protein for many basic and neutral lipophilic drugs as well as steroid hormones from both endogenous and exogenous origin. One to seven binding sites have been described. Serum concentration of AGP, therefore, is an important determinant that may influence the pharmacokinetics for these drugs (5-6). AGP may also play an important role in anti-inflammation and has an immunomodulatory effect. It has been demonstrated that AGP can inhibit neutrophil migration in sepsis and have a protective effect in severe bacterial infection (7-9). AGP can also inhibit platelet aggregation (10). The glycosylation pattern of AGP is associated with certain pathophysiological states. Its immunomodulatory and anti-inflammatory functions also depend on its carbohydrate composition (11-12). Furthermore, AGP has shown promise as a therapeutic agent. When exogenous AGP is administered to pathological conditions associated with ischemia and reperfusion injury, it is able to significantly reduce apoptosis and inflammation (13).

The Quantikine® Human α1-Acid Glycoprotein Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human AGP in cell culture supernates, serum, plasma, and urine. It contains human AGP purified from plasma and has been shown to accurately quantitate the natural protein. Results obtained using natural human AGP 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 AGP.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human AGP has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any AGP present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human AGP 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 AGP 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.
- If samples generate values higher than the highest standard, further 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 AGP Microplate	893786	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human AGP.	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 AGP Standard	893788	2 vials of human AGP in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.* **Note:** *Human sourced material. See Precautions section.*	Use a new standard for each assay. Discard after use.
Human AGP Conjugate	893787	21 mL of a polyclonal antibody specific for human AGP conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-73	895541	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-20 Concentrate	895346	2 vials (21 mL/vial) of a concentrated buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
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.
- 100 mL and 500 mL graduated cylinders.
- 2-8 °C incubator.
- Test tubes for dilution of standards and samples.

PRECAUTIONS

The AGP standard provided with this kit was prepared from human serum or plasma. The source material was tested at the donor level using FDA licensed methods and found to be non-reactive for anti-HIV-1/2 and Hepatitis B surface antigen. As no testing can offer complete assurance of freedom from infectious agents, this reagent should be handled as if capable of transmitting infection.

AGP is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum and plasma samples require a 10,000-fold dilution. A suggested 10,000-fold dilution is 10 µL of sample + 990 µL of Calibrator Diluent RD5-20 (diluted 1:5)*. Complete the 10,000-fold dilution by adding 10 µL of diluted sample to 990 µL of Calibrator Diluent RD5-20 (diluted 1:5)*.

Urine samples require a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5-20 (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of AGP are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

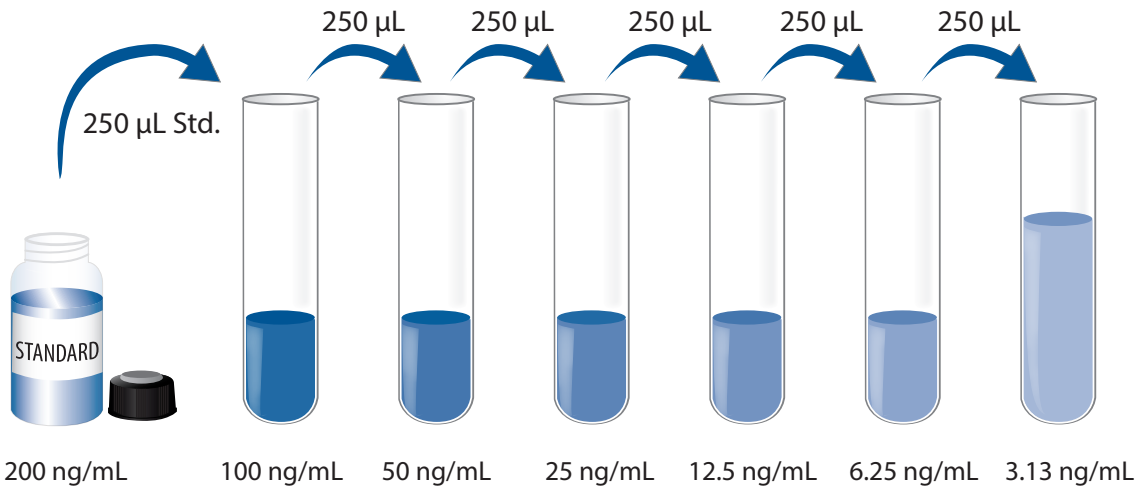
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5-20 (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5-20 Concentrate to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5-20 (diluted 1:5).

Human AGP Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human AGP Standard with Calibrator Diluent RD5-20 (diluted 1:5). This reconstitution produces a stock solution of 200 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.

Pipette 250 µL of Calibrator Diluent RD5-20 (diluted 1:5) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human AGP Standard (200 ng/mL) serves as the high standard. Calibrator Diluent RD5-20 (diluted 1:5) 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: *High concentrations of AGP are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 RD1-73 to each well.
4. Add 50 µL of standard or sample* per well. Cover with the adhesive strip provided. Incubate for 2 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 AGP 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

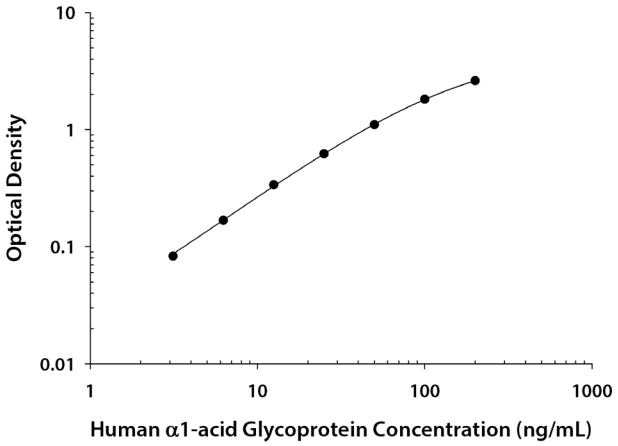
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) 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 the graph. The data may be linearized by plotting the log of the human AGP concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

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	0.048 0.053	0.051	—
3.13	0.133 0.135	0.134	0.083
6.25	0.217 0.220	0.219	0.168
12.5	0.383 0.393	0.388	0.337
25	0.658 0.684	0.671	0.620
50	1.150 1.152	1.151	1.100
100	1.848 1.882	1.865	1.814
200	2.652 2.667	2.660	2.609

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)	16.5	45.8	105	16.7	45.2	105
Standard deviation	0.87	1.96	7.75	1.11	2.96	8.60
CV (%)	5.3	4.3	7.4	6.6	6.5	8.2

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=5)	98	85-111%
Urine* (n=4)	96	86-111%

*Samples were diluted prior to assay.

LINEARITY

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

		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Urine* (n=4)
1:2	Average % of Expected	98	104	107	106	105
	Range (%)	92-108	101-108	104-111	99-110	97-110
1:4	Average % of Expected	99	103	109	101	103
	Range (%)	93-111	100-107	104-114	89-109	93-108
1:8	Average % of Expected	98	103	104	99	107
	Range (%)	91-114	97-106	100-108	86-106	97-115
1:16	Average % of Expected	97	96	102	97	106
	Range (%)	87-114	85-102	94-111	89-106	98-110

*Samples were diluted prior to assay.

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of human AGP ranged from 0.069-0.538 ng/mL. The mean MDD was 0.229 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 highly purified AGP from human serum and plasma produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human AGP in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (µg/mL)	Range (µg/mL)	Standard Deviation (µg/mL)
Serum (n=36)	658	301-1136	208
EDTA plasma (n=36)	628	322-1143	198
Heparin plasma (n=36)	601	286-1087	192

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Urine (n=11)	3060	67.8-19,100	5734

Cell Culture Supernates - HepG2 human hepatocellular carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was removed, assayed for human AGP, and measured 1543 ng/mL.

SPECIFICITY

This assay recognizes natural human AGP.

The factors listed below were prepared at 2.0 mg/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 2.0 mg/mL in a mid-range human AGP control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

C-Reactive Protein
Fetuin A
Serpin A1

Natural proteins:

mouse AGP
human Fibronectin
human Haptoglobin

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