Quantikine® ELISA

Human AgRP Immunoassay

Catalog Number DAGR00

For the quantitative determination of human Agouti-Related Protein (AgRP) concentrations in cell culture supernates, 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

Agouti-Related Protein (AgRP), the protein product of the Agouti-Related Transcript (ART), is a secreted orexigenic (appetite-stimulating) neuropeptide with important roles in the regulation of energy homeostasis via its inhibitory actions on the melanocortin system. Human AgRP is 132 amino acids (aa) with eleven cysteine residues, ten of which are in the mature peptide and form five disulfide bonds (1, 2). Human and murine AgRP are approximately 81% identical at the amino acid level, with the highest identity found in the C-terminal region (1, 2). AgRP shares sequence and structural homology with Agouti, a protein found in skin that regulates coat color in rodents via inhibition of α-melanocyte-stimulating hormone (α-MSH) on melanocortin receptor 1 (MC-1) (3). In contrast, AgRP is localized mainly to the adrenal gland and a subset of neurons in the hypothalamic arcuate nucleus where it acts as an antagonist of α-MSH on MC-3 and MC-4 receptors (2, 4, 5). In addition to AgRP, the orexigenic protein Neuropeptide Y (NPY) is also expressed by this neuronal subset (4, 6).

Studies utilizing animal models suggest that AgRP plays a role in the regulation of energy homeostasis. For instance, AgRP mRNA levels are upregulated by fasting and downregulated by leptin, a key adipose-derived protein important for appetite reduction and increased energy expenditure (4, 7, 8). AgRP is also elevated in leptin-deficient (*ob/ob*) and leptin-insensitive (*db/db*) mice (1). AgRP levels increase in streptozotocin-treated diabetic rats and correlate with accompanying hyperphagia, while AgRP and hyperphagia decrease in response to insulin treatment (9). In addition, transgenic over-expression of AgRP produces an obese phenotype, and exogenous AgRP results in increased feeding behavior and weight gain (2, 10-12). AgRP-induced obesity may result from increased appetite and feeding and/or decreases in metabolic rate (12, 13). RNA interference (RNAi) experiments resulting in a 50% decrease in AgRP mRNA expression are associated with increased metabolic rate and weight loss (14). Both AgRP knockout and AgRP/NPY double knockout mice exhibit no obvious phenotype suggesting the potential for compensatory pathways (15).

AgRP likely plays a role in human energy homeostasis, and several studies suggest that genetic mutations and altered AgRP levels might be associated with appetite regulation and obesity. A single nucleotide polymorphism (SNP; 199 G>A) is associated with anorexia nervosa and late onset obesity (16, 17). An additional SNP (38 C>T) resulting in increased promoter activity is associated with high body mass index (BMI) and type II diabetes in a Western African population (18, 19). In addition, AgRP plasma levels decrease in response to satiety, increase in response to fasting, and are elevated in obese men (20, 21).

The Quantikine® Human AgRP Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human AgRP in cell culture supernates, plasma, and urine. It contains *Sf* 21-expressed recombinant human AgRP and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human AgRP 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 AgRP.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human AgRP has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any AgRP present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human AgRP 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 AgRP 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 the appropriate 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 AgRP Microplate	892541	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human AgRP.	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 AgRP Standard	892543	Recombinant human AgRP in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer. Avoid repeated freeze-thaw cycles.
Human AgRP Conjugate	892542	21 mL of a polyclonal antibody specific for human AgRP conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-35	895271	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-23	895288	21 mL of a buffered protein base with preservatives. *For cell culture supernate/ urine samples.*	
Calibrator Diluent RD6-10	895468	21 mL of a buffered protein base with preservatives. *For plasma samples.*	
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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human AgRP Controls (optional; R&D Systems®, Catalog # QC24).

PRECAUTIONS

Calibrator Diluent RD6-10 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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 MSDS 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.

Note: *Studies performed with matched serum, EDTA plasma, heparin plasma, and recalcified EDTA plasma indicate that some AgRP is destroyed during the clotting process. Therefore, serum is not a valid sample type in this kit.*

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

Plasma - Collect plasma using heparin or EDTA 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

Urine samples require a 2-fold dilution. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5-23.

REAGENT PREPARATION

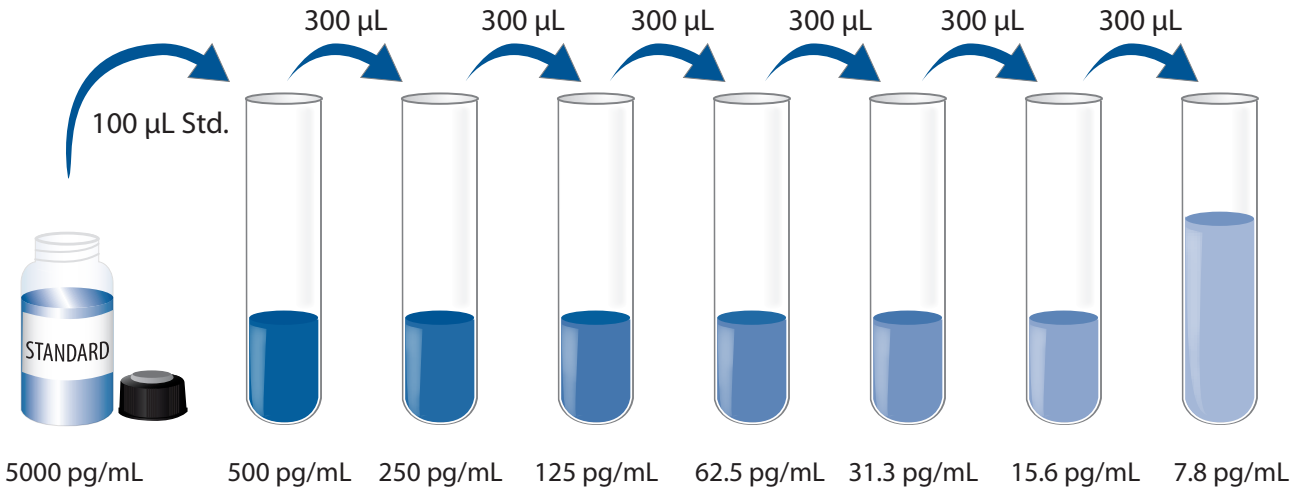
Bring all reagents to room temperature before use.

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 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 AgRP Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human AgRP Standard with deionized or distilled water. This reconstitution produces a stock solution of 5000 pg/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 900 µL of Calibrator Diluent RD5-23 (*for cell culture supernate/urine samples*) or Calibrator Diluent RD6-10 (*for plasma samples*) into the 500 pg/mL tube. Pipette 300 µL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 500 pg/mL standard serves as the high standard. The appropriate calibrator diluent serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

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

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 RD1-35 to each well.
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
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 AgRP Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
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 **on the benchtop. 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.).

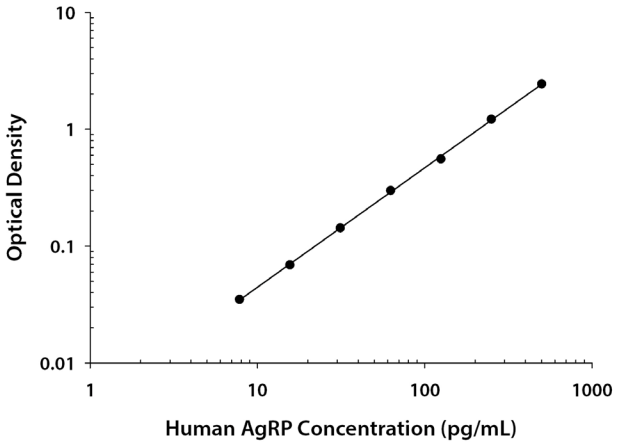
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human AgRP 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

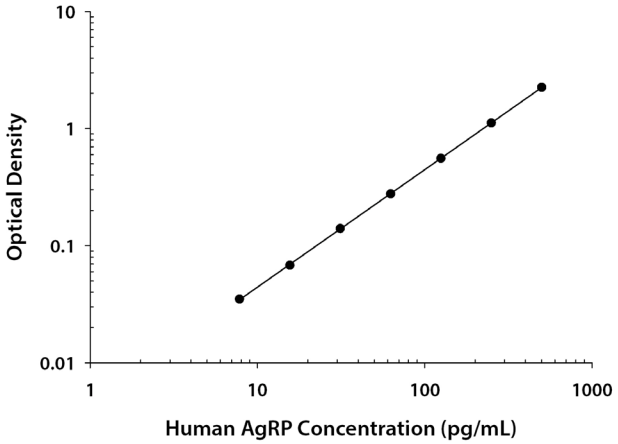
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/URINE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.024 0.026	0.025	—
7.8	0.060 0.060	0.060	0.035
15.6	0.093 0.095	0.094	0.069
31.3	0.167 0.169	0.168	0.143
62.5	0.318 0.329	0.324	0.299
125	0.590 0.601	0.596	0.571
250	1.238 1.245	1.242	1.217
500	2.437 2.492	2.465	2.440

PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.024 0.026	0.025	—
7.8	0.059 0.061	0.060	0.035
15.6	0.090 0.095	0.093	0.068
31.3	0.162 0.168	0.165	0.140
62.5	0.295 0.308	0.302	0.277
125	0.565 0.598	0.582	0.557
250	1.129 1.157	1.143	1.118
500	2.273 2.274	2.274	2.249

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.

CELL CULTURE SUPERNATE/URINE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (pg/mL)	54.3	143	257	43.7	126	238
Standard deviation	2.64	3.88	5.69	3.91	8.24	16.3
CV (%)	4.9	2.7	2.2	8.9	6.5	6.8

PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (pg/mL)	56.6	144	262	45.1	130	246
Standard deviation	3.14	4.86	7.82	4.66	10.0	18.1
CV (%)	5.5	3.4	3.0	10.3	7.7	7.4

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	106	95-115%
EDTA plasma (n=4)	96	89-106%
Heparin plasma (n=4)	92	84-103%

SENSITIVITY

Fifty-four assays were evaluated and the minimum detectable dose (MDD) of human AgRP ranged from 0.17-2.68 pg/mL. The mean MDD was 0.68 pg/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.

LINEARITY

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

		Cell culture media (n=4)	Urine* (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	105	102	107	96
	Range (%)	99-112	95-107	104-111	90-100
1:4	Average % of Expected	104	103	105	102
	Range (%)	99-108	98-109	101-107	94-106
1:8	Average % of Expected	105	102	101	99
	Range (%)	101-109	98-108	99-104	93-109
1:16	Average % of Expected	106	102	99	99
	Range (%)	101-113	98-110	95-103	86-108

*Samples were diluted prior to assay as directed in the Sample Preparation section.

CALIBRATION

This immunoassay is calibrated against a highly purified *Sf* 21-expressed recombinant human AgRP produced at R&D Systems®.

SAMPLE VALUES

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

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
EDTA plasma (n=44)	62.4	35.5 - 115	16.8
Heparin plasma (n=44)	63.3	35.2 - 115	17.5

Urine - Thirty-two samples were evaluated for the presence of human AgRP in this assay. Levels of AgRP measured from non-detectable to 916 pg/mL with a mean value of 455 pg/mL.

Cell Culture Supernates - Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 50 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. Aliquots of the cell culture supernates were removed on days 1 and 5 and assayed for levels of human AgRP. No detectable levels were observed.

SPECIFICITY

This assay recognizes natural and recombinant human AgRP.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human AgRP control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
Agouti (1-40 aa)
ASP (87-132 aa)
GDNF
GFRα-1
GFRα-2
GFRα-3
Neurturin
RET

Recombinant mouse:
GFRα-2
Neurturin
Persephin
RET

Recombinant rat:
GDNF
GDNF Rα-1

Recombinant mouse AgRP (82-131 aa) cross-reacts approximately 13.6%.

Recombinant human AgRP (83-132 aa) cross-reacts approximately 30.0%.

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