



# Human AgRP Immunoassay

**Catalog Number: EA800069**

For the quantitative determination of human AgRP concentrations in cell culture supernates, serum, and plasma.

For research use only. Not for use in diagnostic procedures.

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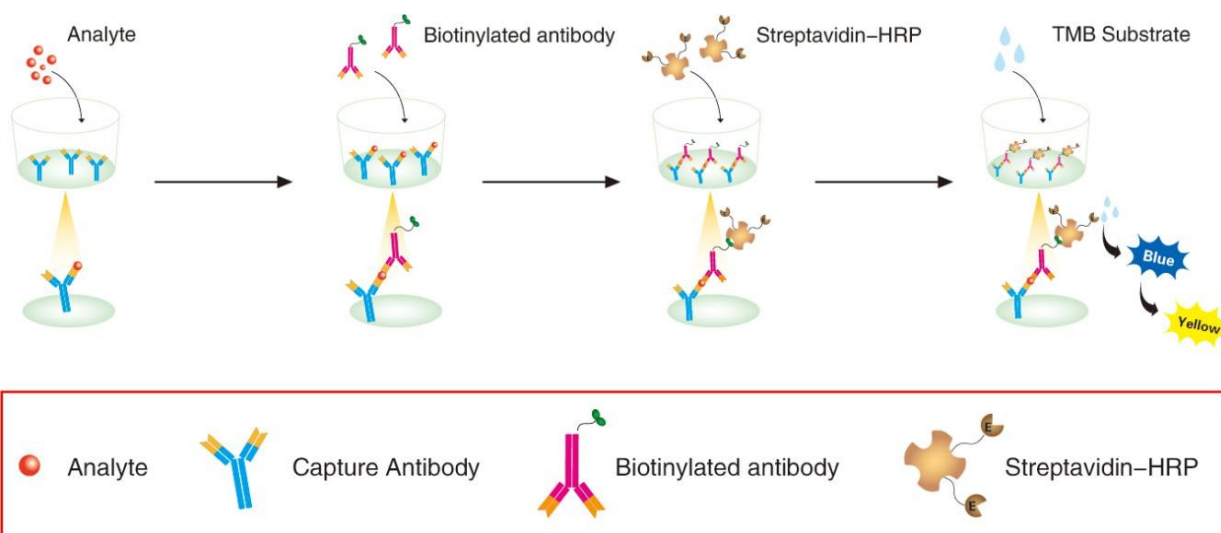
## BACKGROUND

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. Human and murine AgRP are approximately 81% identical at the amino acid level, with the highest identity found in the C-terminal region. AgRP shares sequence and structural homology with Agouti, a protein found in skin that regulates coat color in rodents via inhibition of  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) on melanocortin receptor 1 (MC-1). 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  $\alpha$ -MSH on MC-3 and MC-4 receptors. 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.

## PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for AgRP has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any AgRP present is captured by the coated antibody after incubation. Following extensive washing, a biotin-conjugate antibody specific for AgRP is added to detect the captured AgRP protein in sample. For signal development, horseradish peroxidase (HRP)-conjugated Streptavidin is added, followed by tetramethyl-benzidine (TMB) reagent. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells. Solution containing sulfuric acid is used to stop color development and the color intensity which is proportional to the quantity of bound protein is measurable at 450nm.

### Schematic diagram:



## TECHNICAL HINTS AND LIMITATIONS

1. This ELISA should not be used beyond the expiration data on the kit label.



2. To avoid cross-contamination, use a fresh reagent reservoir and pipette tips for each step.
3. To ensure accurate results, some details, such as technique, plasticware and water sources should be emphasized.
4. A thorough and consistent wash technique is essential for proper assay performance.
5. A standard curve should be generated for each set of samples assayed.
6. It is recommended that all standards and samples be assayed in duplicate.
7. Avoid microbial contamination of reagents and buffers. Buffers containing protein should be made under aseptic conditions and be prepared fresh daily.
8. In order to ensure the accuracy of the results, the standard curve should be made every time.

## PRECAUTIONS

The Stop Solution suggested for use with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

## KIT COMPONENTS& STORAGE CONDITIONS

PART	SIZE	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
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<b>Microwell Plate</b> - antibody coated 96-well Microplate (8 wells ×12 strips)	1 plate	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**
<b>Standard</b> - lyophilized,4000 pg/ml upon reconstitution	2 vials	Aliquot and Store at -20°C** for six months
<b>Concentrated Biotin-Conjugated antibody</b> (100X) - 120 ul/vial	1 vial	Store at 2-8°C **for six months
<b>Concentrated Streptavidin-HRP solution</b> (100X) - 120 ul/vial	1 vial	Store at 2-8°C** for six months
<b>Standard /Sample Diluent</b> - 16 ml/vial	1 bottle	Store at 2-8°C** for six months
<b>Biotin-Conjugate antibody Diluent</b> - 16 ml/vial	1 bottle	Store at 2-8°C** for six months
<b>Streptavidin-HRP Diluent</b> - 16 ml/vial	1 bottle	Store at 2-8°C** for six months
<b>Wash Buffer Concentrate</b> (20x) - 30 ml/vial	1 bottle	Store at 2-8°C** for six months
<b>Substrate Solution</b> - 12 ml/vial	1 bottle	Store at 2-8°C** for six months
<b>Stop Solution</b> - 12 ml/vial	1 bottle	Store at 2-8°C** for six months
<b>Plate Cover Seals</b>	4 pieces	

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

## OTHER SUPPLIES REQUIRED BUT NOT SUPPLIED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Pipettes and pipette tips.

3. Deionized or distilled water.
4. Squirrt bottle, manifold dispenser, or automated microplate washer.
5. 500 mL graduated cylinder.

## SPECIMEN COLLECTION & STORAGE

**Cell Culture Supernates** - Centrifuge cell culture media at 1000×g to remove debris. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

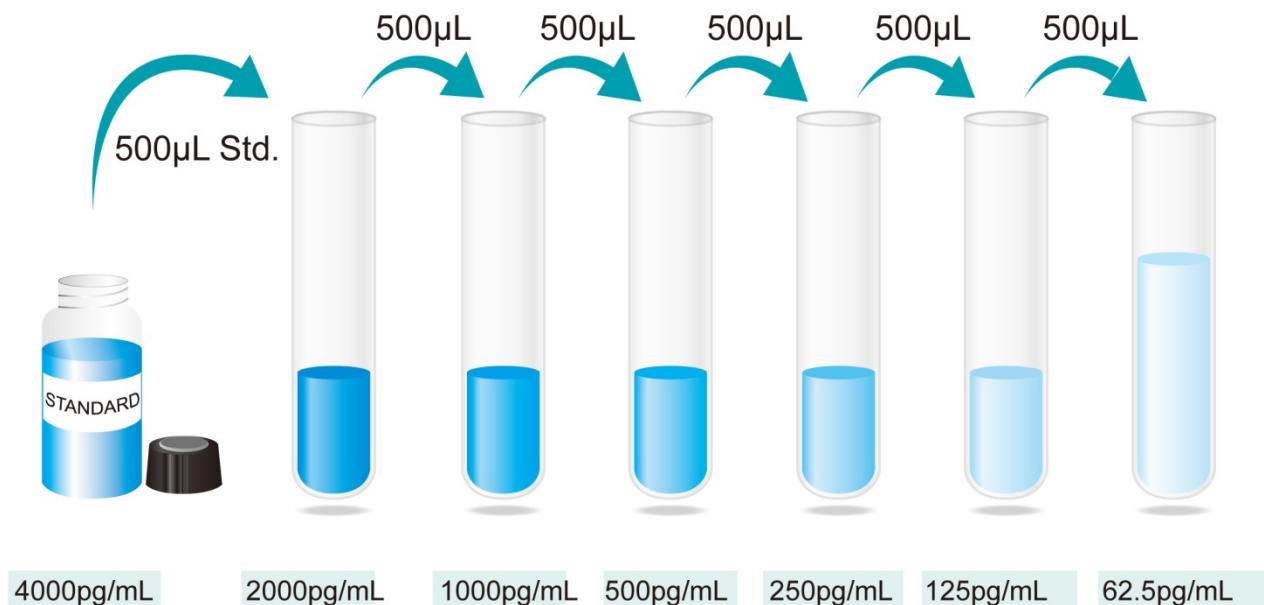
**Serum** - Use a serum separator tube (SST) and allow samples to clot for 2 hours at room temperature or overnight at  $2-8^{\circ}\text{C}$ . Centrifuge approximately for 15 minutes at 1000×g. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Plasma** - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000×g within 30 minutes of collection. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Note: The normal human serum or plasma samples are suggested to make a 1:2 dilution.**

## REAGENTS PREPARATION

1. **Temperature returning** - Bring all kit components and specimen to room temperature ( $20-25^{\circ}\text{C}$ ) before use.
2. **Wash Buffer** - Dilute 30mL of Wash Buffer Concentrate with 570mL of deionized or distilled water to prepare 600mL of Wash Buffer. If crystals have formed in the concentrate Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
3. **Standard/Specimen** - Reconstitute the Standard with 1.0mL of Standard/Sample Diluent. This reconstitution produces a stock solution of 4000pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 500 $\mu\text{L}$  of Standard/Sample Diluent into 2000pg/ml tube and the remaining tubes. Use the stock solution of 4000pg/mL to produce a 2-fold dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 4000 pg/mL standard serves as the high standard. The Standard/Sample Diluent serves as the zero standard (0 pg/mL).



### Preparation of AgRP standard dilutions

**\*If you do not run out of re-melting standard, store it at -20°C. Diluted standard shall not be reused.**

- 4. Working solution of Biotin-Conjugate anti-human AgRP antibody:** Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with the Biotin-Conjugate antibody Diluent in a clean plastic tube.

**\*The working solution should be used within one day after dilution.**

- 5. Working solution of Streptavidin-HRP:** Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with the Streptavidin-HRP Diluent in a clean plastic tube.

**\*The working solution should be used within one day after dilution.**

## ASSAY PROCEDURE

Prepare all reagents and standards as directed. Wash the plate 3 times before assay.



Add 100µl standard or samples to each well, incubate 90 minutes, 37°C.



Aspirate and wash 4 times

Add 100µl working solution of Biotin-Conjugate anti-human AgRP antibody to each well, incubate 60 minutes, 37°C.



Aspirate and wash 4 times

Add 100µl working solution of Streptavidin-HRP to each well, incubate 30 minutes, 37°C.

↓ Aspirate and wash 5 times

Add 100µl Substrate solution to each well, incubate 15 minutes, 37°C. Protect from light.

↓

Add 50µl Stop solution to each well. Read at 450nm within 5 minutes.

## CALCULATION OF RESULTS

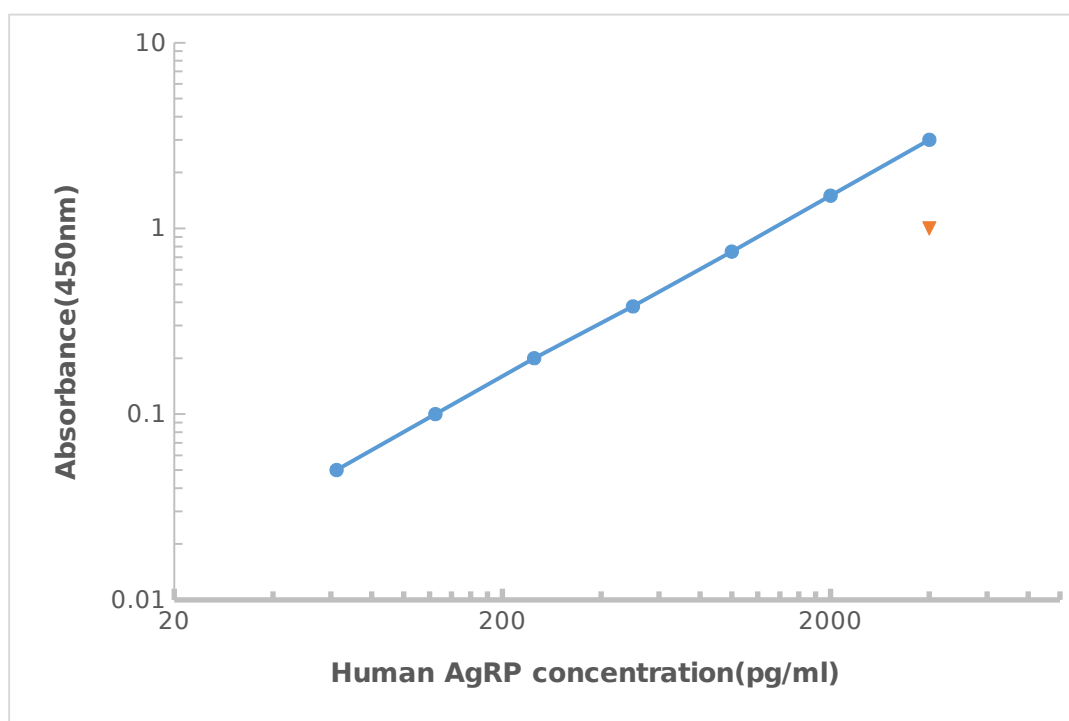
1. The standard curve is used to determine the amount of specimens.
2. First, average the duplicate readings for each standard, control, and sample. All O.D. values are subtracted by the mean value of blank control before result interpretation.
3. Construct a standard curve by reducing the data using 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.
4. The data may be linearized by plotting the log of the AgRP 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.
5. This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

**Typical data using the AgRP ELISA**

Standard (pg/ml)	OD.	OD.	Average	Corrected
0	0.034	0.035	0.034	---
62.5	0.067	0.07	0.068	0.034
125	0.093	0.102	0.097	0.063
250	0.186	0.193	0.189	0.155
500	0.367	0.384	0.375	0.341



1000	0.721	0.752	0.736	0.702
2000	1.387	1.403	1.395	1.360
4000	2.436	2.462	2.449	2.414



**Representative standard curve for AgRP ELISA.**

## Performance Characteristics

**SENSITIVITY:** The minimum detectable dose was 30pg/mL.

**SPECIFICITY:** This assay recognizes both natural and recombinant human AgRP. The factors listed below were prepared at 100ng/ml in Standard /sample Diluent and assayed for cross-reactivity and no significant cross-reactivity or interference was observed.

### Factors assayed for cross-reactivity

Recombinant human	Recombinant mouse	Recombinant porcine
Aaouti (1-40aa)	GFR $\alpha$ -2	
ASP (87-132aa)	Neurturin	
GDNF	Persephin	
GFR $\alpha$ -1	RET	
GFR $\alpha$ -2		
GFR $\alpha$ -3		
Neurturin		
RET		

**REPEATABILITY:** The coefficient of variation of both intra-assay and inter-assay were less than 10%.

**RECOVERY:** The recovery of AgRP spiked to three different levels in four samples throughout the range of the assay in various matrices was evaluated.

#### Recovery of AgRP in two matrices

Sample Type	Average % of Expected Range (%)	Range (%)
Citrate plasma	91	83-102
Cell culture supernatants	94	86-103

**LINEARITY:** To assess the linearity of the assay, three samples were spiked with high concentrations of AgRP in various matrices and diluted with the appropriate Sample Diluent to produce samples with values within the dynamic range of the assay. (The plasma samples were initially diluted 1:1)

Dilution ratio	Recovery (%)	Citrate plasma	Cell culture supernatants
1:2	Average% of Expected	96	103

	Range (%)	88-103	95-112
1:4	Average% of Expected	98	105
	Range (%)	87-106	97-113
1:8	Average% of Expected	95	101
	Range (%)	89-103	91-108
1:16	Average% of Expected	102	107
	Range (%)	95-110	98-115

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