



Human Amylin
96-Well Plate Assay
Cat. # EZHA-52K

HUMAN AMYLIN ELISA KIT
96-Well Plate (Cat. # EZHA-52K)

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HUMAN AMYLIN ELISA KIT
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I. INTENDED USE

This kit is for non-radioactive quantification of Human Amylin in plasma. The capture antibody requires an intact disulfide bond between positions 2 and 7 of the peptide. One kit is sufficient to measure 38 unknown samples in duplicate. ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE

The Human Amylin ELISA is a monoclonal antibody-based sandwich immunoassay for determining amylin levels in human plasma. The capture antibody recognizes Human Amylin, Amylin Acid (deamidated amylin), a 1-20 fragment of amylin, but not reduced amylin. The detection antibody binds to reduced or unreduced Human Amylin but not Amylin Acid and is complexed with Streptavidin-Alkaline Phosphatase. The substrate, 4-Methylumbelliferyl Phosphate (MUP), is applied to the completed sandwich and the fluorescent signal, monitored at 355 nm/460 nm, is proportional to the amount of amylin present in the sample.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well microtiter plate and contains the following reagents:

A. Human Amylin ELISA Plate

Coated with Mouse anti-Human Amylin Antibody
Quantity: 1 plate
Preparation: Ready to use

B. Adhesive Plate Sealer

Quantity: 1 Sheet
Preparation: Ready to use

C. 10X TBS Wash Buffer Concentrate

10X concentrate of 50 mM Tris Buffered Saline with Tween 20 and Sodium Azide
Quantity: 50 mL
Preparation: Dilute 1:10 with deionized water

D. Human Amylin Standard

Human Amylin in Assay Buffer: 100 pM
Quantity: Lyophilized, 1 mL /vial rehydrated
Preparation: Reconstitute with 1 mL deionized water

E. ELISA Amylin Quality Controls 1 and 2

Human Amylin in Assay Buffer.
Quantity: Lyophilized, 250 µL /vial rehydrated
Preparation: Reconstitute with 250 µL deionized water

F. Assay Buffer

0.05M PBS, pH 7.4, containing Proprietary Protease Inhibitors, with Tween 20,
0.08% Sodium Azide and 1% BSA
Quantity: 12 mL / vial
Preparation: Ready to use

III. REAGENTS SUPPLIED

G. Human Amylin Detection Conjugate

Anti-Human Amylin-Alkaline Phosphatase Conjugate

Quantity: 11 mL

Preparation: Ready to use

H. Substrate (Light sensitive, avoid unnecessary exposure to light)

4-Methylumbelliferyl Phosphate

Quantity: 10 mg

Preparation: Hydrate in 1 mL deionized water just before use. Use at 1:200 dilution in substrate diluent (e.g. 105 μ L hydrated substrate in 21 mL substrate diluent).

I. Substrate Diluent (Light sensitive, avoid unnecessary exposure to light)

Quantity: 21 mL

Preparation: Ready to use, warm to room temperature before use.

J. Stop Solution

Quantity: 6 mL

Preparation: Bring to room temperature before use. Mix thoroughly to ensure no precipitate remains.

IV. STORAGE AND STABILITY

Upon receipt, all components of the kit should be stored at 2-8°C. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTIONS

A. Diethanolamine

Substrate diluent contains diethanolamine. This compound can be harmful through ingestion, inhalation, and skin contact. May be irritating to eyes and skin. If skin/eye contact occurs flush thoroughly with water.

B. Sodium Azide

Sodium Azide has been added to reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipet with Tips, 10 μ L-200 μ L
2. Multi-Channel Pipette, 50 μ L-300 μ L
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Absorbent Paper or Cloth
6. Refrigerator
7. Deionized Water
8. Orbital Microtiter Plate Shaker
9. Fluorescence Plate Reader

VII. SAMPLE COLLECTION AND STORAGE

1. For plasma collection, collect whole blood in ice-cooled Vacutainer® EDTA-plasma tubes. Centrifuge immediately at 1000 xg for 10 minutes in refrigerated centrifuge or place tubes on ice and centrifuge within one hour.
2. Specimens should be stored at less than or equal to $\leq -70^{\circ}\text{C}$. Aliquot samples before freezing if necessary.
3. Avoid using samples with gross hemolysis or lipemia.

VIII. STANDARD AND QUALITY CONTROLS PREPARATION

A. Human Amylin Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human Amylin Standard with 1.0 mL distilled or deionized water into the glass vial to give a 100pM concentration of Standard. Invert and mix gently, let sit for 5 minutes then vortex gently.
2. Label six tubes 50, 25, 12.5, 6.25, 3.125, 1.56 pM. Add 0.5 mL Assay Buffer (Sample Diluent) to each of the six tubes. Prepare serial dilutions by adding 0.5 mL of the 100 pM reconstituted standard to the 50 pM tube, mix well and transfer 0.5 mL of the 50 pM reconstituted standard to the 25 pM, mix well and transfer 0.5 mL of the 25 pM Standard to the 12.5 pM tube, mix well and transfer 0.5 mL of the 12.5 pM Standard to the 6.25 pM tube, mix well and transfer 0.5 mL of the 6.25 pM Standard to the 3.125 pM tube, mix well and transfer 0.5 mL of the 3.125 pM Standard to the 1.56 pM tube, mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at $\leq -20^{\circ}\text{C}$. Avoid multiple freeze/thaw cycles.

Standard Concentration pM	Volume of Deionized Water to Add	Volume of Standard to Add
100	1.0 mL	

Standard Concentration pM	Volume of Assay Buffer (Samples Diluent) to Add	Volume of Standard to Add
50	0.5 mL	0.5 mL of 100 pM
25	0.5 mL	0.5 mL of 50 pM
12.5	0.5 mL	0.5 mL of 25 pM
6.25	0.5 mL	0.5 mL of 12.5 pM
3.125	0.5 mL	0.5 mL of 6.25 pM
1.56	0.5 mL	0.5 mL of 3.125 pM

B. Human Amylin Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the Human Amylin Quality Control 1 and Quality Control 2 with 0.25 mL distilled or deionized water into the glass vials. Invert and mix gently, let sit for 5 minutes then mix well.

IX. ASSAY PROCEDURE

The assay should be run in duplicate using 50 μ L Assay Buffer and 50 μ L of Standard, Control, or Sample in each well.

1. Dilute the concentrated Wash Buffer 10 fold by mixing the entire contents of the 10X Wash Buffer with 450 mL deionized water.
2. Remove the microtiter assay plate from the foil pouch and fill each well with 300 μ L of diluted TBS Wash Buffer. Incubate at room temperature for 10 minutes, no shaking.
3. Decant Wash Buffer from the plate and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Do not let wells dry before proceeding to the next step.
4. Add 50 μ L Assay Buffer to each well.
5. Add in duplicates; 50 μ L Assay Buffer to reference tubes, 50 μ L Standards, Samples and Controls. Refer to Section IX for suggested well orientations. Seal plate and incubate at room temperature on the shaker for one hour. (**NOTE: Start incubation time as plate is loaded on the shaker, not from the time you start loading the plate with samples.**) Decant and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
6. Wash the plate 3 times with 300 μ L per well Wash Buffer. Decant and tap after each wash to remove residual buffer.
7. Add 100 μ L Detection Conjugate to each well. Cover the plate with sealer and incubate on the shaker at room temperature for 2 hours.
8. Near the completion of this incubation step, hydrate the Substrate (ESS-MUP) by adding 1 mL deionized water to 10 mg, mix well, and let stand 15 minutes (with occasional mixing) to assure complete dissolution. Remove 105 μ L from the reconstituted substrate and add it to the 21 mL vial of Substrate Diluent (EDD-MUP), mix well. Referred to as Substrate Solution from here on.
9. Decant Detection Conjugate and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
10. Wash the plate 3 times with 300 μ L per well Wash Buffer. Decant and tap after each wash to remove residual buffer.
11. Add 100 μ L Substrate Solution to each well. Incubate at least 20 minutes at room temperature in the dark.
12. Read plate on a fluorescent plate reader with an excitation/emission wavelength of 355nm/460nm. Monitor to see if there is significant signal-to-noise ratio with the lowest point on standard curve (i.e. 1.56 pM), and the highest standard point (i.e. 100 pM) within the maximum relative fluorescence unit (RFU) read-out of plate reader. Incubate longer if necessary.
13. If sufficient fluorochrome has been generated, add 50 μ L Stop Solution to each well in the same order as the Substrate was added, and read on the Fluorescence Plate reader after 5 minutes.

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X. MICROTITER PLATE ARRANGEMENT

	1	2	3	4	5	6	7	8	9	10	11	12
A	0pM	0 pM	1.56 pM	1.56 pM	3.125 pM	3.125 pM	6.25 pM	6.25 pM	12.5 pM	12.5 pM	25 pM	25 pM
B	50 pM	50 pM	100pM	100pM	QC 1	QC 1	QC 2	QC 2	Sample 1	Sample 1	Sample 2	Sample 2
C	Etc.											
D												
E												
F												
G												
H												

XI. CALCULATIONS

The RFU can be fitted directly to the concentration. If curve fitting software is available, the best fit can be obtained with a linear-linear spline fit.

Since this assay is a direct ELISA, the RFU is directly proportional to the concentration of Human Amylin in the sample.

Note: When sample volumes assayed differ from 50 μ L, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 25 μ L of sample is used, then calculated data must be multiplied by 2).

XII. ASSAY CHARACTERISTICS

Sensitivity

The lowest level of Human Amylin that can be detected by this assay is 1 pM (50 μ L plasma sample size).

Performance

$$ED_{80} = 84 \pm 2 \text{ pM}$$

$$ED_{50} = 60 \pm 4 \text{ pM}$$

$$ED_{20} = 32 \pm 3 \text{ pM}$$

Crossreactivity

Human Glucagon	<1%
Human GLP-1	<1%
Human Insulin	<1%
Human Pancreatic Polypeptide	<1%
Human Adrenomedullin	1%
Human Calcitonin	<1%
Calcitonin Gene Related Peptide	<1%

Note: This kit is suitable for the measurement of Amylin in rat and feline plasma; however, the precise percent of cross-reactivity is not determined at this time.

XII. ASSAY CHARACTERISTICS (continued)

Precision

Within and Between Assay Variation

Sample No.	Amylin Added pM	Within % CV	Between % CV
1	20	1.8	5.9
	50	1.6	6.0
	80	1.2	3.7
2	20	2.2	4.9
	50	1.2	6.1
	80	1.9	3.7
3	20	1.7	4.6
	50	3.4	6.9
	80	1.9	4.8

The assay variation of Millipore Human Amylin ELISA kits were studied at three different spiked concentrations of Amylin in three different Human Plasma samples. The within variation is the mean from four duplicate determinations in a single assay. The between variation is the mean value of the mean of four duplicate determinations in each plasma across six assays.

Recovery

Spike & Recovery of Human Amylin in Human Plasma

Sample #	Sample Concentration (pM)	Amylin Added (pM)	% of Recovery
1	6.14	20	92
		50	93
		80	94
2	5.75	20	97
		50	96
		80	96
3	6.85	20	99
		50	99
		80	97

Varying concentrations of Human Amylin were added to three Human Plasma samples and the amylin content was determined in six different ELISA assays. The % of Recovery = observed amylin concentration/expected amylin concentration x 100%.

XII. ASSAY CHARACTERISTICS (continued)

Linearity

Effect of Plasma Dilution

Sample No.	Volume Sampled	Expected pM	Observed pM	% Of Expected
1	50 μ L	27.9	27.9	100
	40 μ L		27.2	97
	25 μ L		30.7	110
	10 μ L		33.1	118
2	50 μ L	16.6	16.6	100
	40 μ L		16.9	102
	25 μ L		16.1	97
	10 μ L		10.7	64
3	50 μ L	33.4	33.3	100
	40 μ L		32.6	98
	25 μ L		25.2	76
	10 μ L		22.6	68

Three Human Plasma samples with the indicated sample volumes were assayed in six different assays. Required amount of Assay Buffer was added to compensate for lost volumes below 50 μ L. The resulting dilution factors of 1.0, 1.25, 2.0, and 5.0 representing 50 μ L, 40 μ L, 25 μ L, and 10 μ L sample volumes assayed, respectively, were applied in the calculation of observed amylin concentrations.

$\% \text{ expected} = \text{observed/expected} \times 100\%$.

XIII. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com.

XIV. TROUBLESHOOTING GUIDE

Low or No Signal with Standards

- * Insufficient time for reaction with substrate. Allow substrate to react longer.
- * Kit reagents have expired.
- * Inadequate plate washing after sample incubation.
- * Too much washing after conjugate incubation can reduce signal.

High Background

- * Inadequate plate washing. After conjugate incubation, tap out plate on absorbent towels after decanting.
- * Plate was not kept in dark after substrate addition.
- * Cross contamination between neighboring wells.
- * Substrate has been diluted too long or exposed to light before use, or diluent has been contaminated with old substrate.

Samples too High

- * Dilute sample with Assay Buffer to bring Human Amylin concentration within standard range.

Signal too High on Highest Standard

- * Plate incubated too long with substrate. Discard substrate, wash plate once and add freshly prepared substrate. Check RFU in less time.

High Variance in RFU of Duplicates

- * Cross contamination in wells.
- * Bubbles in substrate at time of reading.
- * Loss of reagent or faulty pipetting in duplicates.

XV. REPLACEMENT REAGENTS

Reagents	Cat. #
Human Amylin ELISA Plate	EP52
10X TBS Wash Buffer Concentrate	EWB-TR
Human Amylin Standards	E8051-K
ELISA Amylin Quality Controls 1 & 2	E6051
Assay Buffer	AB-A
Human Amylin Detection Conjugate	E1051
Substrate	ESS-MUP
Substrate Diluent	EDD-MUP
Stop Solution	ET-AP

XVI. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:

Toll Free US (800) MILLIPORE

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to human or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.

XVII. REFERENCES

1. Tijssen P. "Practice and Theory of Enzyme Immunoassays" in Burdon RH and Knippenberg PH (Ed.), Laboratory Techniques in Biochemistry and Molecular Biology. Amsterdam/NY: Elsevier, 1985
2. Christopoulos TK and Diamandis EP. "Fluorescence Immunoassays" in Diamandis EP and Christopoulos TK (Ed.), Immunoassay. Academic Press, 1996
3. Percy A, Rittenhouse J, Trainor D, Phelps J, and Koda J.: Development of Sensitive Immunoassays to Detect Amylin and Amylin-Like Peptides in Untreated Plasma. *Clinical Chemistry* 42:4 , pp 576-585
4. Phelps, et al., "Development and Characterization of Monoclonal Antibodies Specific for Amylin" *Hybridoma*. Vol 15 No 5, pp 379-386