User Guide

Human Amylin

96-Well Plate

EZHA-52K
EZHA-52BK

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

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.

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Principles of Assay

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.

Reagents Supplied

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

Note: Store all reagents at 2-8 °C.

Reagents Supplied	Volume	Quantity	Cat. No.
Human Amylin ELISA Plate with 2 plate sealers	-	1 plate 2 sealers	EP52
Human Amylin Standard	1 mL Lyophilized	1 vial	E8051-K
ELISA Amylin Quality Controls 1 and 2	250 µL each Lyophilized	1 vial each	E6051-K
Assay Buffer	12 mL	1 bottle	AB-A
10X TBS Wash Buffer Concentrate	50 mL	1 bottle	EWB-TR
Human Amylin Detection Conjugate	11 mL	1 bottle	E1051
Substrate Diluent	21 mL	1 bottle	EDDMUP-AMLN
Substrate Solution (**Light Sensitive:** avoid unnecessary exposure to light)	10 mg	1 bottle	ESSMUP-AMLN
Stop Solution (**Caution:** Corrosive Solution)	6 mL	1 bottle	ETAP-AMLN

Storage and Stability

Recommended storage for kit components is 2-8 °C.

All components are shipped and stored at 2-8 °C. Reconstituted standards and controls can be frozen for future use but repeated freeze/thaw cycles should be avoided. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

Reagent Precautions

Sodium Azide

Sodium azide or Proclin™ has been added to some reagents as a preservative. Although the concentrations are low, Sodium azide and Proclin™ may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

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.

Note: See next page for Hazardous Component full labels.

Symbol Definitions

Ingredient	Cat. No.	Full Label	
ELISA Amylin Quality Controls 1 and 2	E6051-K		**Warning.** Harmful if swallowed. Causes serious eye irritation. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Human Amylin Standard	E8051-K		**Warning.** Harmful if swallowed. Causes serious eye irritation. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Substrate Diluent	EDDMUP-AMLN		**Danger:** Causes skin irritation. Causes serious eye damage. May cause damage to organs through prolonged or repeated exposure if swallowed. Wear eye protection. IF ON SKIN: Wash with plenty of soap and water. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention if you feel unwell.

Materials Required (Not Provided)

- Multi-channel Pipettes and pipette tips: 50 µL-300 µL
- Pipettes and pipette tips: 10 µL-200 µL
- Buffer and Reagent Reservoirs
- Vortex Mixer
- Deionized water
- Refrigerator
- Orbital Microtiter Plate Shaker
- Absorbent Paper or Cloth
- Fluorescence plate reader

Sample Collection and Storage

1. For plasma collection, collect whole blood in ice-cooled Vacutainer® EDTA-plasma tubes.
2. Invert tube several times to mix, immediately add protease inhibitor cocktail for Amylin measurement. We recommend our Protease Inhibitor Cocktail following manufacturer's instructions.
3. Centrifuge immediately at 1000 x *g* for 10 minutes in refrigerated centrifuge or place tubes on ice and centrifuge within one hour.
4. Specimens should be stored at less than or equal to < −70 °C. Aliquot samples before freezing if necessary.
5. Avoid using samples with gross hemolysis or lipemia.

Standard and Quality Controls Preparation

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 vial. Invert and mix gently, let sit for 5 minutes then vortex gently.
2. Label six tubes 1, 2, 3, 4, 5, and 6. Add Assay Buffer to each of the six tubes according to the volumes outlined in the chart below. Dilute the reconstituted standard stock according to the chart below. Vortex each tube briefly to ensure complete mixing.

   Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of reconstituted standard should be stored at ≤ −20 °C. Avoid multiple freeze/thaw cycles.

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration pM
1.0 mL	-	X (Refer to analysis sheet for exact concentration)

Tube #	Volume of Assay Buffer to Add	Volume of Standard to Add	Standard Concentration (ng/mL)
1	500 µL	500 µL of reconstituted standard	X/2
2	500 µL	500 µL of tube 1	X/4
3	500 µL	500 µL of tube 2	X/8
4	500 µL	500 µL of tube 3	X/16
5	500 µL	500 µL of tube 4	X/32
6	500 µL	500 µL of tube 5	X/64

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 vials. Invert and mix gently, let sit for 5 minutes then mix well.

Assay Procedure

The assay should be run in duplicate using 50 µL Assay Buffer and 50 µL 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 µL 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 µL Assay Buffer to each well.
5. Add in duplicates; 50 µL Assay Buffer to reference tubes, 50 µL Standards, Samples and Controls. Refer to Microtiter Plate Arrangement 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 µL per well Wash Buffer. Decant and tap after each wash to remove residual buffer.
7. Add 100 µL 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 (ESSMUP-AMLN) 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 µL from the reconstituted substrate and add it to the 21 mL bottle of Substrate Diluent (EDDMUP-AMLN), 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 µL per well Wash Buffer. Decant and tap after each wash to remove residual buffer.

11. Add 100 µL Substrate Solution to each well. Incubate at least 20 minutes at room temperature in the dark.

    Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

12. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. Read plate on a fluorescent plate reader with an excitation/emission wavelength of 355 nm/460 nm. Monitor to see if there is significant signal-to-noise ratio with the lowest point on standard curve (for example, 1.56 pM), and the highest standard point (for example, 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 µL Stop Solution to each well in the same order as the Substrate was added, and read on the Fluorescence Plate reader after 5 minutes.

Microtiter Plate Arrangement

Human Amylin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 3 Standard	QC 1	Etc.								
B	Blank	Tube 3 Standard	QC 1	Etc.								
C	Tube 6 Standard	Tube 2 Standard	QC 2									
D	Tube 6 Standard	Tube 2 Standard	QC 2									
E	Tube 5 Standard	Tube 1 Standard	Sample 1									
F	Tube 5 Standard	Tube 1 Standard	Sample 1									
G	Tube 4 Standard	Reconstituted Standard	Sample 2									
H	Tube 4 Standard	Reconstituted Standard	Sample 2									

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 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (for example, if 25 µL of sample is used, then calculated data must be multiplied by 2).

Assay Characteristics

Sensitivity

The lowest level of Human Amylin that can be detected by this assay is 0.7 pM (50 µL plasma sample size).

Performance

ED_{80} = 84 ± 2 pM

ED_{50} = 60 ± 4 pM

ED_{20} = 32 ± 3 pM

Cross-Reactivity

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.

Precision

Sample #	Amylin Added (pM)	Assay Variation (%CV)	
		Intra-assay	Inter-assay
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 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.

Spike and 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 100%.

Effect of Plasma Dilution

Sample #	Volume Sampled (µL)	Expected (pM)	Observed (pM)	% of Expected
1	50	27.9	27.9	100
	40		27.2	97
	25		30.7	110
	10		33.1	118
2	50	16.6	16.6	100
	40		16.9	102
	25		16.1	97
	10		10.7	64
3	50	33.4	33.3	100
	40		32.6	98
	25		25.2	76
	10		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 µL. The resulting dilution factors of 1.0, 1.25, 2.0, and 5.0 representing 50 µL, 40 µL, 25 µL, and 10 µL sample volumes assayed, respectively, were applied in the calculation of observed amylin concentrations.

% expected = observed/expected x 100%

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert, or available at our website SigmaAldrich.com.

Troubleshooting

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.

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Cat. No.
Microtiter Plate	EP52
10X TBS Wash Buffer Concentrate	EWB-TR
Human Amylin Standards	E8051-K
ELISA Amylin Quality Controls 1 and 2	E6051-K
Assay Buffer	AB-A
Human Amylin Detection Conjugate	E1051
Substrate Diluent	EDDMUP-AMLN
Substrate	ESSMUP-AMLN
Stop Solution	ETAP-AMLN
10 pack of Human Amylin ELISA Kits	EZHA-52BK

References

1. Tijsen 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

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