DRG® Total Human Amylin (EIA-3741)

Revised 11 June 2007 **RUO**

INTENDED USE

This kit is for non-radioactive quantification of Total Human Amylin in plasma. The capture antibody recognizes an epitope near the midpoint of the peptide. One kit is sufficient to measure 38 unknown samples in duplicate. ***This kit is for research purposes only.***

PRINCIPLES OF PROCEDURE

The Total Human Amylin ELISA is a monoclonal antibody-based sandwich immunoassay for determining total amylin levels in human plasma. The capture antibody recognizes reduced human amylin and human amylin acid (deamidated amylin), but not the 1-20 fragment of 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 microtiter plate and contains the following reagents:

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

2. Adhesive Plate Sealer
Quantity: 1 Sheet
Preparation: Ready to use

3. Wash Buffer Concentrate 10X
10X concentrate of 50 mM Tris Buffered Saline containing Tween 20 and Sodium Azide
Quantity: 50 ml/vial
Preparation: Dilute 1:10 with deionized water

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

5. Quality Controls 1 and 2
Human Amylin in Assay Buffer.
Quantity: 250 µl /vial, Lyophilized
Preparation: Reconstitute with 250 ml deionized water

6. 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

7. Detection Antibody
Anti Human Amylin-Alkaline Phosphatase Conjugate
Quantity: 11 ml/vial
Preparation: Ready to use

8. Substrate (Light sensitive, avoid unnecessary exposure to light)
4-Methylumbelliferyl Phosphate
Quantity: 10 mg

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

9. Substrate Diluent (Light sensitive, avoid unnecessary exposure to light)
Quantity: 21 ml/vial
Preparation: Ready to use, warm to room temperature before use

10. Stop Solution
Quantity: 6 ml
Preparation: Bring to room temperature before use. Mix thoroughly to ensure no precipitate remains.

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.

REAGENT

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.

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 large volume of water to prevent azide builds up.

MATERIALS REQUIRED BUT NOT PROVIDED

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

SAMPLE COLLECTION AND STORAGE

1. For plasma collection, collect whole blood in ice-cooled Vacutainer EDTA-plasma tubes. Centrifuge immediately at 1000-x g for 10 minutes in refrigerated centrifuge or place tubes on ice and centrifuge within one hour.
2. Specimens should be stored at ≤ 70°C. Aliquot samples before freezing if necessary.
3. Avoid using samples with gross hemolysis or lipemia.

STANDARD AND QUALITY CONTROL 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 100 pM 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

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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 ≤ -20°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 of12.5 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 Controls with 0.25 ml distilled or deionized water into the glass 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 Wash Buffer. Incubate at room temperature for 10 minutes, no shaking.
3. Decant Wash Buffer 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 of Assay Buffer to reference tubes, 50 µl 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 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 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 vial of Substrate Diluent, mix well. Referred to as Substrate Solution from here on.

9. Decant Detection Antibody 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 15 minutes at room temperature in the dark, no shaking.
12. Read plate on a fluorescent plate reader with an excitation/emission wavelength of 355nm/460nm. Note the RFU of the top standard point; when the reading is 2000 RFU or greater, add 50 µl Stop Solution, gently mix, and read on the Fluorescence Plate reader after 5 minutes.

MICROTITER PLATE ARRANGEMENT

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pM	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	100 pM	100 pM	QC1	QC1	QC2	QC2	Sample1	Sample1	Sample2	Sample2
C	Etc.											
D												
E												
F												
G												
H												

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 Total 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 (e.g., if 25 µl of sample is used, then calculated data must be multiplied by 2).

ASSAY CHARACTERISTICS

SENSITIVITY

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

CROSSREACTIVITY

Total Human Amylin	100%
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 not suitable for the determination of Total Amylin levels in rat or feline plasma.

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PRECISION

Within and Between Assay Variation

Sample No.	Mean pM	Within % CV	Between % CV
1	20	2.6	11.9
	50	5.0	12.19
	80	14.8	11.5
2	20	11.5	17.4
	50	2.5	17.8
	80	7.9	16.2
3	20	7.8	17.6
	50	5.4	11.9
	80	7.3	13.0

The assay variation of DRG Human Amylin (total) ELISA kits were studied at three different 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 Total Human Amylin in Human Plasma

Sample No.	Sample Conc. (pM)	Amylin Added (pM)	% of Recovery
1	3.25	20	104
		50	99
		80	91
2	2.56	20	96
		50	89
		80	89
3	11.23	20	101
		50	87
		80	90

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%.

LINEARITY

Effect of Plasma Dilution

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Sample No.	Volume Sampled	Expected pM	Observed pM	Percent expect.
1	50 µl	22.32	22.32	100
	40 µl		21.13	95
	25 µl		19.98	90
	10 µl		18.55	83
2	50 µl	25.34	25.35	100
	40 µl		22.30	88
	25 µl		20.54	81
	10 µl		21.05	83
3	50 µl	20.54	20.54	100
	40 µl		20.85	101
	25 µl		15.96	78
	10 µl		19.65	96

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%.

Troubleshooting Guide

1. 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.

2. 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.

3. Samples too high

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

4. 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.

5. 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

REFERENCES

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