Instruction For Use

NEOLISA™ Chromogranin A

Enzyme immunoassay for detection of chromogranin A

Microtitration 96 wells
Store the kit at +2-8 °C

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For Research Use Only. Not for use in diagnostic procedures.

REF **CGA RUO**

Σ 96

PURPOSE OF RESEARCH PRODUCT

The NEOLISA™ Chromogranin A test kit is an enzyme-linked immunosorbent assay (ELISA) for detection of chromogranin A in human plasma or serum in test subjects with signs and symptoms consistent for NETs. The result shall not be used for clinical diagnosis or patient management.

SUMMARY AND EXPLANATION

Chromogranins and secretogranins constitute a family of uniquely acidic proteins that are co-stored with neurotransmitters and peptide hormones in the brain and the diffuse neuroendocrine system (Winkler, H. & Fischer-Colbrie, R.1992). Structurally these proteins are products of different genes but share some overall properties such as an abundance of acidic amino acid residues and several pairs of basic amino acids as potential positions for post-translational cleavage. Chromogranins are co-stored and co-released with neuropeptides and hormones in the neuroendocrine cells throughout the body. A role for chromogranins in the generation of hormonal granules and package of hormones has been suggested. Furthermore, chromogranins can be cleaved into smaller fragments, which can display biological activities such as inhibition of hormonal release, vasodilatation and anti-microbiological effects (Stridsberg M, 2000).

Tumours of neuroendocrine origin usually present with increased serum/plasma levels of chromogranin A (O'Connor, DT, Deftos LJ, 1986). The neuroendocrine tumours are derived from the neuroendocrine cells and typical neuroendocrine tumours are carcinoid tumours, pheochromocytomas, neuroblastomas, small cell lung cancers, hyperparathyroid adenomas, pituitary tumours and pancreatic islet tumours and including the MEN1 and MEN2 syndromes. This also includes the different neuroendocrine tumour syndromes, namely the gastrinomas, insulinomas, glucagonomas, somatostatinomas. PPomas and the non-functioning neuroendocrine tumours (Eriksson, B. et al. 2000). For these tumours, chromogranin A has been shown to be the best circulating marker (Bajetta, E. et al. 1999).

Principle of NEOLISA™ Chromogranin A

In a separate dilution plate samples, calibrators and controls are diluted 5x in Diluent. The diluted material is transferred to the microtitre wells and incubated at room temperature for 60 minutes. During this first incubation a monoclonal antibody captures the chromogranin A to surface of the well. After washing to remove unbound material a second, horseradish peroxidase (HRP) labelled monoclonal antibody is added to detect the chromogranin A bound to the well. After incubation for 30 minutes the wells are washed again and a colour substrate is added and incubated. The colour development is stopped after 15 minutes and the colour measured in a spectrophotometer. The colour is directly proportional to the amount of chromogranin A bound to the well. The amount of chromogranin is determined by comparison with the colour development of the calibrator samples.

The calibrator in the kit is a synthetic peptide corresponding to chromogranin A. The peptide calibrator is set to give a response equal to a purified, native fragment of chromogranin A (Stridsberg, M et al. 1993, Stridsberg et al. 1995)

WARNINGS AND PRECAUTIONS

For research use only – not for use in clinical procedures.

- The assay reagents contain no human serum components but the plasmas analysed should be handled as if capable of transmitting infectious agents.
- The Centers for Disease Control and Prevention and National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.
- Most of solutions contain ProClin 300 as a preservative. Never pipette by mouth or allow reagents or sample to come into contact with skin. Reagents containing ProClin 300 may be irritating. Avoid contact with skin and eyes. In case of contact, flush with plenty of water.

- The stop solution contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin.
- The concentrations of chromogranin A in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.
- All the waste should be handled as hazardous wastes.
- Material safety data sheets for all hazardous components contained in this kit are available on request from Euro Diagnostica.

Warning

CAL	X	LYO
DIL		
CONJ	100X	

CONTROL	L	LYO
CONTROL	H	LYO

Contains ProClin 300:
Reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC no. 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC no. 220-239-6] (3:1)

H317:	May cause an allergic skin reaction.
P264:	Wash hands thoroughly after handling.
P280:	Wear protective gloves/protective clothing/eye protection/face protection.
P302+352:	IF ON SKIN: Wash with plenty of soap and water.
P333+313:	If skin irritation or rash occurs: Get medical advice/attention.

SPECIMEN COLLECTION

The NEOLISA™ Chromogranin A is recommended for serum/EDTA/Heparin-plasma.
Handle as if capable of transmitting infectious agents.
The samples are separated by centrifugation and can be stored at 2-8 °C up to 7 days. If samples are to be kept for longer periods, store at -20 °C or colder. Samples may go through 3 freeze/thaw cycles without deterioration.
Do not use a frost-free freezer because it may allow the specimens to go through freeze-thaw cycles and degrade antibody. Samples that are improperly stored or are subjected to multiple freeze-thaw cycles may yield spurious results.

KIT COMPONENTS AND STORAGE OF REAGENTS

- One frame with 96 wells, sealed in a foil pack with a dry pack.
- Six vials with lyophilised calibrators containing human Chromogranin A in diluent. CAL 1 = 0 ng/mL (diluent), CAL 2 = 36 ng/mL, CAL 3 = 180 ng/mL, CAL 4 = 540 ng/mL, CAL 5 = 1080 ng/mL, CAL 6 = 1800 ng/mL.
- One vial with lyophilised Low control (L), green colour .
- One vial with lyophilised High control (H), red colour.
- 30 mL Diluent (DIL) containing biotin-labelled antibodies to Chromogranin A, red colour. Ready to use.
- 150 µL Conjugate (CONJ) containing HRP-labelled antibodies to Chromogranin A. 100x concentrated.
- 15 mL Conjugate buffer (blue colour). Ready to use.
- 15 mL Substrate TMB. Ready to use.
- 15 mL Stop solution (0.5M H_2SO_4). Ready to use.
- 2 x 35 mL Wash solution, 20x concentrated.

Before use
Lyophilised calibrators and controls should be carefully dissolved with 300 µL distilled water. After use the reconstituted calibrators and controls should be stored at -20 °C or lower. Calibrators and controls may go through three freeze/thaw cycles without deterioration.
Other reagents in the kit should be stored at 2-8 °C.
Reconstituted calibrators and controls should be diluted 5x in diluent immediately prior to setting up a test. The amount of conjugate needed for each analysis should be diluted 100x before use (10uL conjugate + 990 uL conjugate buffer per strip).. Any leftover diluted conjugate must be discarded.
Wash solution should be diluted 20x before use. Dilute 10 mL of the 20x concentrated wash solution in 190 mL distilled water. When stored at 2-8 °C, the diluted wash solution is stable until the date of expiration of the kit.
The rest of the reagents in the kit are ready for use.
Remove only the number of wells needed for testing, reseal the aluminium packaging carefully.

Materials or equipment required but not provided
- Microplate reader with 450 nm filter. Reference wavelength is 620 nm.
- 300 µL/well dilution plate for dilution of calibrator, unknown samples and controls.
- Precision pipettes with disposable tips.
- Automatic microtitre plate washer, absorbent tissue, tubes and a timer.

PROCEDURE
All solutions should be used at room temperature. Incubate all steps at room temperature (20-30 °C).

Sample dilution and incubation
Dilute all samples 5x in a separate dilution plate before transferring to test plate. Calibrators, Low control, High control and unknown samples should all be diluted 50 µL plasma + 200 µL diluent. Mix thoroughly before transferring 100µL in duplicate to the test plate according to the diagram below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CAL1	CAL5	P1									
B	CAL1	CAL5	P1									
C	CAL2	CAL6	P2									
D	CAL2	CAL6	P2									
E	CAL3	H	etc									
F	CAL3	H										
G	CAL4	L										
H	CAL4	L										

Incubate for 60 minutes.

After sample incubation
Wash three (3) times with 300 µL washing solution/well, filling and emptying the wells each time. After the last wash, empty the wells by tapping the inverted microtitre plate sharply on the absorbent tissue.

Adding conjugate
Immediately add 100 µL conjugate to each well.
Incubate for 30 minutes.

After conjugate incubation
Wash as before.

Adding substrate solution
Immediately add 100 µL substrate TMB to each well, incubate in the dark for 15 minutes. The incubation time may be shortened to 10 minutes if maximum Optical Density (OD) exceeds 3.0 at high temperatures or if an automated method is applied.

Adding stop solution
Add 100 µL stop solution to each well. Read the absorbance at 450 nm within 2h on a microplate reader. Read at 620 nm as a reference wavelength.

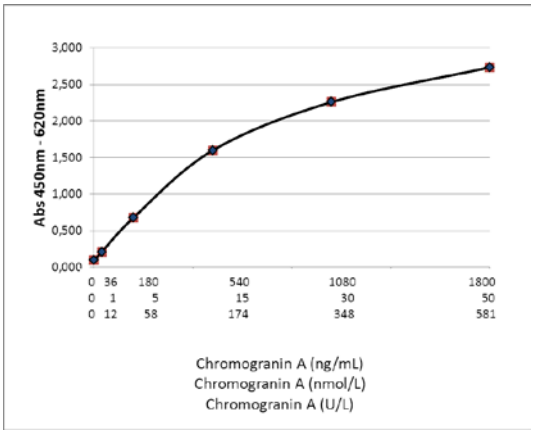
Calculations
Construct a calibrator curve by plotting the OD against the nmol/L values of the six calibrators. The six calibrators provided have values of 0 ng/mL for calibrator 1, 36 ng/mL for calibrator 2, 180 ng/mL for calibrator 3, 540 ng/mL for calibrator 4, 1080 ng/mL for calibrator 5, 1800 ng/mL for calibrator 6 respectively. Read the values of Low and High controls and the unknown samples from the curve. Values greater than the highest calibrator value should be reported as >1800 ng/mL, or diluted further with assay diluent and re-assayed. In this case the compensation for the dilution must be made in the calculation of the chromogranin A concentration.

Please note: in the event of calibrator 1 or calibrator 6 values are out of range the test should be considered invalid and repeated.

For convenience concentrations can be calculated in ng/mL, nmol/L or U/L. All units are given in the table below. The molecular weight of the native fragment of chromogranin A (36kDa) was used for the conversion (Stridsberg, M et al. 1993, Stridsberg et al. 1995)

Example

Calibrator	Concentration			Absorbance
	ng/mL	nmol/L	U/L	
1	0	0	0	0.094
2	36	1	12	0.209
3	180	5	58	0.678
4	540	15	174	1.599
5	1080	30	348	2.259
6	1800	50	581	2.735



A sample with an absorbance value of 1.272 will read on the X-axis as having 367 ng/mL (or 10.2 nmol/L, or 118 U/L) of chromogranin A. In this example a four parameter logistic curve fit has been applied.
Important: The curve is an example and should not be used for actual sample interpretation.

Quality Control
The OD for calibrator 1 should be be <0.15.
The OD for calibrator 6 should be >1.0
The values of Low and High controls, see lot certificate.
The controls are intended to monitor for substantial reagent failure. If any of the control values are not within their respective ranges, the test should be considered invalid and should be repeated. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organisations. Refer to NCCLS C24-A for guidance on appropriate QC practices.

Hook-effect
No hook-effect is observed when testing chromogranin A concentrations up to 360 000 ng/mL (or 10 000 nmol/L, or 116 000 U/L).

LIMITATIONS
The individual chromogranin A level cannot alone be used as a measure of disease severity.
Always interpret laboratory test results in conjunction with other indicators.

Interference
No interference was detected when plasma samples were spiked with bilirubin F (208 mg/L) , bilirubin C (200 mg/L), haemolytic haemoglobin (4,84 g/L) or chyle (1430 FTU).
Individuals receiving mouse anti-human antibodies for treatment or diagnosis, or those who have been otherwise exposed to mouse immunoglobulin, may produce human anti-mouse antibodies (HAMA). These antibodies can interfere with assays using mouse monoclonal antibodies and may cause falsely elevated levels.

Expected results
It is recommended that each laboratory establishes a reference range with samples commonly used since variations in sample handling may affect results. A strict sample handling routine as recommended above should be followed. The values given below are an indication of the expected reference range and calculated as the 97.5 percentile of 126 blood donor Heparin-plasma samples (63 men and 63 women).
Reference range level of chromogranin A: ≤ 108 ng/mL (or 3.0 nmol/L. or 35 U/L).

Limit of Detection
The limit of detection of this assay is 19 ng/mL (or 0.54 nmol/L, or 6 U/L). This is the lowest detectable concentration that differs from zero with a probability of 95%. It means that reliable measurements can be done even on negative samples down to this point. The calculation was done according to NCCLS guideline EP17-A vol. 24 no. 34.

Non-tumour associated increases of chromogranin A
Increased levels of chromogranin A can be found in subjects with decreased renal function, atrophic gastritis and subjects being treated with proton-pump inhibitory drugs.

PERFORMANCE CHARACTERISTICS
Table 1. Sensitivity and specificity.

Sensitivity
A total of 107 symptomatically characterized Heparin-plasma samples were assayed. The following table summarises the results

Disease groups	Total number	Positive >3.0 nmol/L	Negative ≤ 3.0 nmol/L	Sensitivity %
ECL	4	3	1	75
EPT	30	19	11	63
FGC	10	7	3	70
LC	9	4	5	44
MGC	47	28	19	60
NE Diff	6	2	4	33
Paragangliom	1	1	0	100

ECL = Enterochromaffin-like Tumours
EPT = Endocrine pancreas Tumour
FGC = Forgut carcinoid
LC = Lung carcinoid
MGC = Midgut carcinoid
NE Diff = Neurocrine differentiation

Specificity
126 Heparin-plasma samples from apparently healthy blood donors were assayed, 126 samples were negative:

126/126 = 100% 95% CI = 97.1%-100%

The 95% confidence interval (CI) was calculated using the exact method.

Table 2. Inter-assay precision was determined by testing six different samples in eight replicates at three separate occasions.

	1	2	3	4	5	6
Mean value (ng/mL)	150	209	316	486	838	1212
SD	14.0	10.8	28.3	41.7	80.6	69.8
% CV	9	5	9	9	10	6

Table 3. Intra-assay precision was determined by testing six different samples in eight replicates at one occasion.

	1	2	3	4	5	6
Mean value (ng/mL)	156	214	335	502	868	1255
	6.4	11.3	10.4	23.3	42.8	101.4
	4	5	3	5	5	8

Table 4. Batch to batch variation was determined by testing six different samples in eight replicates on three different batches.

	1	2	3	4	5	6
Mean value (ng/mL)	145	205	309	459	794	1165
	9.6	7.8	22.9	38.5	69.2	78.1
	7	4	7	8	9	7

Table 5. Dilution recovery was determined by testing five serial dilutions for three different samples.

Sample	Dilution	Mean Measured Concentration (ng/mL)	Calculated Concentration (ng/mL)	Dilution Corrected % Recovery
1	1/5	1540	1540	100
	1/10	756	770	98
	1/20	366	385	95
	1/40	179	193	93
	1/80	90	96	94
Sample	**Dilution**	**Mean Measured Concentration (ng/mL)**	**Calculated Concentration (ng/mL)**	**Dilution Corrected % Recovery**
2	1/5	1134	1134	100
	1/10	587	567	104
	1/20	278	283	98
	1/40	139	142	98
	1/80	72	71	101
Sample	**Dilution**	**Mean Measured Concentration (ng/mL)**	**Calculated Concentration (ng/mL)**	**Dilution Corrected % Recovery**
3	1/5	1293	1293	100
	1/10	625	646	97
	1/20	292	323	90
	1/40	144	162	89
	1/80	76	81	94

TROUBLESHOOTING

Problem	Possible causes	Corrective action
One or more calibrators out of range.	1. One or more reagents not added, or added in wrong sequence.	1. Test invalid. Repeat test.
	2. Improper pipetting.	2. Test invalid. Repeat test.
Control values out of range.	1. Incorrect temperature, timing or pipetting; reagents not mixed.	1. Check that the time and temperature was correct. See ”Poor precision” below. Repeat test.
	2. Cross contamination of controls.	2. Pipette carefully.
	3. Improper dilution.	3. Repeat test.
	4. Optical pathway not clean.	4. Check for dirt or air bubbles in the wells. Wipe bottom of the plate and reread.
All test Results blank.	1. One or more reagents not added, or added in wrong sequence.	1. Recheck procedure. Check for unused reagent. Repeat test.
	2. Coated plate inactive.	2. Check for obvious moisture in unused wells. Wipe bottom of the plate and reread.
All test results yellow.	1. Contaminated buffers or reagents.	1. Check all solutions for turbidity or foul smell.
	2. Wash solution contaminated.	2. Use clean container. Check quality of water solution used to prepare.
	3. Improper dilution of serum.	3. Repeat test.
Maximum OD is elevated (exceeds 3.0)	1. Too high incubation temperatures/use of automated method.	1. Reduce the incubation time for substrate.
Poor precision.	1. Pipette delivery CV higher than 5%.	1. Check calibration of pipette. Use reproducible technique.
	2. Sample or reagents not mixed sufficiently or not equilibrated to room temperature.	2. Mix all reagents gently but thoroughly and equilibrate to room temperature.
	3. Reagent addition taking too long; inconsistency in timing intervals.	3. Develop consistent uniform technique and use multi-tip device or auto dispenser to decrease time.
	4. Optical pathway not clean.	4. Check for air bubbles in the wells. Wipe bottom of the plate and reread.
	5. Washing not consistent; trapped bubbles; wash solution left in the wells.	5. Check that all wells are filled and aspirated uniformly. Dispense liquid above level of reagent in well. After the last wash, empty the wells by tapping the plate on an absorbent tissue.
	6. Improper pipetting.	6. Avoid air bubbles in pipette tips.

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EXPLANATION OF SYMBOLS.

	Batch code.
	Catalogue number.
	Use-by date.
	Temperature limit.
	Biological risks.
	Consult instructions for use.
	Manufacturer.
	Warning.
	Contains sufficient for 96 tests.

Ag	Antigen (coated plate).
DIL	Diluent.
BUF **CONJ**	Conjugate buffer.
CONJ **100X**	Conjugate HRP 100x conc.
BUF **WASH** **20X**	Wash buffer 20x concentrate.
SUBS **TMB**	Solution TMB (substrate solution).
H_2SO_4 **0.5M**	Sulphuric Acid, 0.5 molar (stop solution).
CAL **X** **LYO**	Lyophilised calibrator.
CONTROL **L** **LYO**	Lyophilised Low control.
CONTROL **H** **LYO**	Lyophilised High control.

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