Instruction

EURIA-Chromogranin A

Chromogranin A radioimmunoassay

Document No. E-23-0045-17 RUO

July, 2013

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

REF **RB 321 RUO**

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INTRODUCTION

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.

Tumours of neuroendocrine origin usually present with increased serum/plasma levels of chromogranin A. 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, prostate cancers 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).

The first radioimmunoassay for measurements of chromogranin A was introduced in 1986 (O'Connor, D.T. & Deftos, L.J. 1986). Since then other assays for measurements of intact human chromogranin A have been reported. Assays for measurements of defined regions of chromogranin A have also been established, such as specific methods for pancreastatin and other regions of chromogranin A (Stridsberg, M. 2000).

The present chromogranin A is a competitive method based on polyclonal antibodies raised in rabbits. The antibodies were raised against a purified fragment containing amino acid sequence 116-439 in the chromogranin A molecule.

PRINCIPLE OF THE METHOD

The intended use of these reagents is the determination of chromogranin A in human serum or plasma. The basic principle for determination of chromogranin A with the EURO-DIAGNOSTICA chromogranin A RIA kit is competitive radioimmunoassay using antibodies against human chromogranin A.
Chromogranin A in standards and samples compete with ^{125}I-labelled chromogranin A in binding to the antibodies. The ^{125}I-chromogranin A binds to the antibodies in an inverse proportion to the concentration of chromogranin A in standards and samples. Antibody-bound ^{125}I-chromogranin A is separated from the unbound fraction using the double antibody solid phase technique. The bound fraction of ^{125}I-chromogranin A is measured in a gamma counter.
For professional use within a laboratory. The result shall not be used for clinical diagnosis or patient management.

PHYSIOLOGICAL CONSIDERATIONS

Tumours of neuroendocrine origin usually present with increased serum/plasma levels of chromogranin A. The neuroendocrine tumours are derived from the neuroendocrine cells. Typical neuroendocrine tumours are carcinoid tumours, pheochromocytomas, neuroblastomas, small cell lung cancers, hyperparathyroid adenomas, pituitary tumours, prostate cancers and pancratic 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.

Reference range, serum: ≤ 3.0 nmol/L.
The reference range was set by testing 127 blood donors (63 men and 64 women, ages 20-68 years). The upper range ≤ 3.0 nmol/L was calculated as the 97.5 percentile.

It is recommended that users establish reference ranges for the populations served by their own laboratories.

Non-tumour associated increases of chromogranin A
Increased levels of chromogranin A can be seen in individuals with decreased renal function, atrophic gastritis and with ongoing treatment with proton-pump inhibitory drugs.

PRECAUTIONS

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

As the regulations may vary from one country to another, it is essential that the person responsible for the laboratory is familiar with current local regulations, concerning all aspects of radioactive materials of the type and quantity used in this test.

This kit contains components of human origin. They have been tested by immunoassay for hepatitis B surface antigen, antibodies to HCV and for antibodies to HIV-1 and HIV-2 and found to be negative. Nevertheless, all recommended precautions for the handling of blood derivatives should be observed.

This kit contains ^{125}I (half-life: 60 days), emitting ionizing X (28 keV) and γ (35.5 keV) radiations. Steps should be taken to ensure the proper handling of the radioactive material, according to local and/or national regulations. Only authorized personnel should have access to the reagents.

The following precautions should be observed when handling radioactive materials:

- Radioactive material should be stored in specially designated areas, not normally accessible to unauthorized personnel.

- Handling of radioactive material should be conducted in authorized areas only.

- Care should be exercised to prevent ingestion and contact with the skin and clothing. Do not pipette radioactive solutions by mouth.

- Drinking, eating or smoking should be prohibited where radioactive material is being used.

- Hands should be protected by gloves and washed after using radioactive materials.

- Work should be carried out on a surface covered by disposable absorbing material.

- Spills of radioactive material should be removed immediately, and all contaminated materials disposed as radioactive waste. Contaminated surfaces should be cleaned with a detergent.

The reagents in this kit contain sodium azide. Contact with copper or lead drainpipes may result in the cumulative formation of highly explosive azide deposits. On disposal of the reagents in the sewerage, always flush with copious amounts of water, which prevents metallic azide formation. Plumbing suspected of being contaminated with these explosive deposits should be rinsed thoroughly with 10% sodium hydroxide solution.

COMPOSITION OF THE REAGENT KIT

The reagents provided in this kit are sufficient for 100 tubes.

1. Anti-chromogranin A (Reagent A)
Rabbit antiserum to human chromogranin A (amino acids 116-439). The antiserum is diluted and lyophilized in 2.0 mL 0.25 M phosphate buffer, pH 7.4, with 1.0% bovine serum albumin, 0.375 M NaCl, 0.25% NaN_3 and 2.5% Tween 20.
Colour: Yellow. Reconstitution in 11.0 mL distilled water.

2. ^{125}I-Chromogranin A (Reagent B)
Activity: 56 KBq (1.5 μCi) on activity reference date. Lyophilized in 2.5 mL 0.25 M phosphate buffer, pH 7.4, with 1.0% bovine albumin, 0.375 M NaCl, 0.25% NaN_3 and 2.5% Tween 20.
Colour: Blue. Reconstitution in 12.5 mL distilled water.

3. Double antibody solid phase (Reagent C)
Anti-rabbit-Ig coupled to cellulose particles in 0.0375 M phosphate buffer pH 7.4 with 0.15% Bovine serum albumin, 0.05625 M NaCl, 0.0375% NaN_3 and 0.375% Tween 20.
52 mL suspension.

4. Assay diluent (Reagent D)
50 mL of 0.05 M phosphate buffer, pH 7.4, with 0.2% bovine serum albumin, 0.075 M NaCl, 0.05% NaN_3 and 0.5% Tween 20. Buffer used for dilution of samples, preparation of working standards and for replacement of antiserum in non-specific binding controls.

5. Chromogranin A standard (Reagent E)
Concentration: 10.0 nmol/L
Volume: 2.00 mL standard after reconstitution.
Lyophilized in 2.00 mL 0.05 M phosphate buffer, pH 7.4 with 0.2% bovine serum albumin, 0.075 M NaCl, 0.05% NaN_3 and 0.5% Tween 20.
Reconstitution in 2.00 mL distilled water.

6. Controls (Reagent F-G)
Lyophilized controls with two different levels of chromogranin A. 1.00 mL of each control after reconstitution. The chromogranin A concentrations are given on the labels of the vials. The controls should not be diluted after reconstitution.
Reconstitution in 1.00 mL distilled water.

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

Distilled water.

Disposable test tubes 11-13 x 55 mm, (polystyrene).

Pipettes with disposable tips, 50, 100 and 500 μL.

Volumetric pipettes 1.00 and 5.00 mL

Vortex mixer.

Centrifuge, refrigerated, minimum g-force 1700 x g.

Gamma counter.

REAGENT PREPARATION AND STORAGE

Store all reagents at 2-8° C before reconstitution and use. The water used for reconstitution of the lyophilized reagents should be distilled in an all-glass apparatus or be of corresponding purity. Dissolve the contents in the vials by gentle inversion and avoid foaming. The stability of the reagents is found on the labels of the vials. For lyophilized reagents the expiry date is valid for the unreconstituted reagents. Reconstituted reagents are stable for 12 weeks.

Reagent A: Anti-chromogranin A
Reconstitute with 11.0 mL distilled water.
Store at 2-8° C.

Reagent B: ^{125}I-Chromogranin A
Reconstitute with 12.5 mL distilled water.
Store at -20° C or lower if reused.

Reagent C: Double antibody solid phase
Ready for use. Stir continuously during pipetting this reagent.
Store at 2-8° C.

Reagent D: Assay diluent
Ready for use.
Store at 2-8° C.

Reagent E: Chromogranin A standard
Reconstitute with 2.00 mL distilled water.
Store at -20° C or lower if reused.

Reagent F-G: Controls
Reconstitute each vial with 1.00 mL distilled water.
Store at -20° C or lower if reused.

SPECIMEN COLLECTION

Veinous blood is collected in tubes without additives or in tubes containing Heparin (144 U.S.P. Heparin in a 10 mL tube), EDTA or Lithium. The samples are cooled in an ice-bath. The samples are separated by centrifugation at 2-4° C and stored at -20° C or lower. The samples should be frozen at –20° C within three hours from sample collection.

ASSAY PROCEDURE

Reconstitute the reagents as specified. The reagents should be brought to room temperature prior to use.

Accuracy in all pipetting steps is essential. All tests (standards, controls and samples) should be performed in duplicate.

For an overview see page 12.

A complete assay includes:

Standards (St-tubes): 7 different concentrations, 0, 0.156, 0.313, 0.625, 1.25, 2.50 and 5.00 nmol/L.

Controls (C-tubes): Low and high.

Samples (P-tubes)

Tubes for determination of the ***non-specific binding (NSB-tubes)***

Tubes for determinatin of the ***total radioactivity*** added ***(TOT-tubes).***

Dilution of samples
Samples should be diluted 1:10 with the assay diluent (Reagent D) before assay.
Samples with chromogranin A concentrations more than 50 nmol/L can be diluted further with assay diluent, and re-assayed.

PERFORMANCE

1. Reconstitute the lyophilized reagents according to the instructions on page 6 and allow the reagents to reach room temperature.

2. Prepare the chromogranin A working standards by dilution of the chromogranin A standard 10.00 nmol/L (Reagent E) with assay diluent (Reagent D) according to the following:
   a. 0.40 mL standard 10.00 nmol/L + 0.40 mL assay diluent = 5.00 nmol/L
   b. 0.40 mL standard 5.00 nmol/L + 0.40 mL assay diluent = 2.50 nmol/L
   c. 0.40 mL standard 2.50 nmol/L + 0.40 mL assay diluent = 1.25 nmol/L
   d. 0.40 mL standard 1.25 nmol/L + 0.40 mL assay diluent = 0.625 nmol/L
   e. 0.40 mL standard 0.625 nmol/L + 0.40 mL assay diluent = 0.313 nmol/L
   f. 0.40 mL standard 0.313 nmol/L + 0.40 mL assay diluent = 0.156 nmol/L
   g. Assay diluent = 0 nmol/L
   Store the standards at –20° C or lower if reused.

3. Dilute the samples 1:10 with assay diluent e.g. 50 µL sample and 450 µL assay diluent. Vortex-mix carefully.

4. Pipette 100 µL of standards (0-5.00 nmol/L) controls and samples in their respective tubes.

5. Pipette 100 µL of zero-standard (assay diluent) in the NSB-tubes.

6. Pipette 100 µL ^{125}I-chromogranin A (Reagent B) in all tubes. The TOT-tubes are sealed and kept aside.

7. Pipette 100 µL anti-chromogranin A (Reagent A) in all tubes except the NSB-tubes and TOT-tubes.

8. Pipette 100 µL assay diluent (Reagent D) in the NSB-tubes.

9. Vortex-mix all tubes carefully.

10. Incubate for 20-24 hours at 2-8° C,

11. Pipette 500 µL double antibody solid phase (Reagent C) in all tubes except the TOT-tubes. This reagent should be stirred continuously with a magnetic stirrer during pipetting. Vortex-mix carefully.

12. Incubate for 30-60 minutes at 2-8° C.

13. Centrifuge for 15 minutes at +4° C (minimum 1700 x g).

14. Decant the supernatants.

15. Count the radioactivity of the pellet in all tubes in a gamma counter.
    Counting time: 1-3 minutes.

CALCULATION

1. Subtract the average count rate (CPM) of the NSB-tubes from the count rate (CPM) of the standards, controls and samples.

2. A standard curve is generated by plotting the bound fraction CPM or B/TOT against the concentrations of the chromogranin A standards. An example of a standard curve is given on page 13.

3. Interpolate the chromogranin A concentrations of the controls and samples from the generated standard curve. Multiply the found concentrations in the samples with the dilution factor 10 (or actual dilution factors if further dilution has been done).

4. The standard curve and the calculation of the chromogranin A concentrations in samples and controls can also be done by a computer method.

QUALITY CONTROL

In order to enable the laboratory to completely monitor the consistent performance of the assay, the following important factors should be checked.

1. Controls
The found concentrations of the controls (Reagent F-G) should be within the limits given on the labels of the vials.

2. Total counts
Counts obtained should approximate the expected CPM when adjusted for counter efficiency and radioactive decay. The content of ^{125}I-chromogranin A in this kit will give a total counts in the assay (TOT) of 21.000 CPM (-10, +20%) at the activity reference date (counting efficiency = 80%).

3. Maximum binding (Bo/TOT)
Calculate for each assay the % bound radioactivity in the zero-standard: $\frac{Bo}{TOT} \times 100\%$.

$\frac{Bo}{TOT} \times 100$ is generally 30-45% at the activity reference date.

$\frac{Bo}{TOT} \times 100$ may have decreased a few % at the expiry date of the kit.

4. Non-specific binding (NSB/TOT)
Calculate for each assay the non-specific binding $\frac{NSB}{TOT} \times 100$.

$\frac{NSB}{TOT} \times 100$ is less than 6% if decanting is made properly.

5. Shape of standard curve
For example, monitor the 80, 50 and 20% points of the standard curve for run to run reproducibility.

PERFORMANCE CHARACTERISTICS

Clinical sensitivity

A total of 43 Heparin-plasma samples with clinical characterisation were assayed. The following table summarises the results (20 men and 23 women, ages 29-91 years)

Disease groups	Total number	Positive >3.0 nmol/L	Negative ≤ 3.0 nmol/L	Sensitivity %
EPT	11	7	4	64
FGC	8	8	0	100
MGC	21	21	0	100
FEO	2	2	0	100
NEC	1	0	1	0

EPT = Endocrine Pancreas Tumour
FGC = Forgut Carcinoid
MGC = Midgut Carcinoid
FEO = Pheochromocytoma
NEC = Neuroendocrine carcinoma

Specificity

127 serum samples from apparently healthy blood donors were assayed, 125 were negative:

125/127 = 98.4% 95% CI = 94.4-99.8%

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

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

	1	2	3	4	5	6
Mean value (nmol/L)	2.9	7.8	19.8	23.9	61.4	75.9
SD	0.1	0.5	1.0	1.4	2.1	2.4
% CV	4.1	6.0	5.0	5.7	3.9	3.2

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 (nmol/L)	2.9	7.5	18.6	23.3	58.7	73.7
SD	0.2	0.6	1.3	1.1	2.3	3.3
% CV	5.3	7.3	6.8	4.7	3.9	4.5

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 (nmol/L)	3.2	7.5	16.2	5.2	23.3	88.0
SD	0.3	0.3	0.8	0.6	1.4	2.3
% CV	10.7	4.3	5.1	10.6	6.1	2.6

Interference

Samples displaying cloudiness, hemolysis, hyperlipemia or containing fibrin may give inaccurate results.

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

Sample	Dilution	Mean Measured Concentration (nmol/L)	Calculated Concentration (nmol/L)	Dilution Corrected % Recovery
1	1/10	23.2	23.2	100%
	1/20	10.2	11.6	88%
	1/40	5.1	5.8	88%
	1/80	3.2	2.9	110%
	1/160	1.7	1.5	113%
Sample	**Dilution**	**Mean Measured Concentration (nmol/L)**	**Calculated Concentration (nmol/L)**	**Dilution Corrected % Recovery**
2	1/10	56.3	56.3	100%
	1/20	24.3	28.2	86%
	1/40	10.8	14.1	77%
	1/80	5.7	7.0	81%
	1/160	2.8	3.5	80%
Sample	**Dilution**	**Mean Measured Concentration (nmol/L)**	**Calculated Concentration (nmol/L)**	**Dilution Corrected % Recovery**
3	1/10	77.9	77.9	100%
	1/20	42.5	39.0	109%
	1/40	18.7	19.5	96%
	1/80	9.0	9.7	93%
	1/160	4.6	4.9	94%

Limit of Detection

The limit of detection of this assay is 0.35 nmol/L (2 × SD 20 blanks). This is the lowest detectable concentration that differs from zero. It means that reliable measurements can be done on negative samples down to this point.

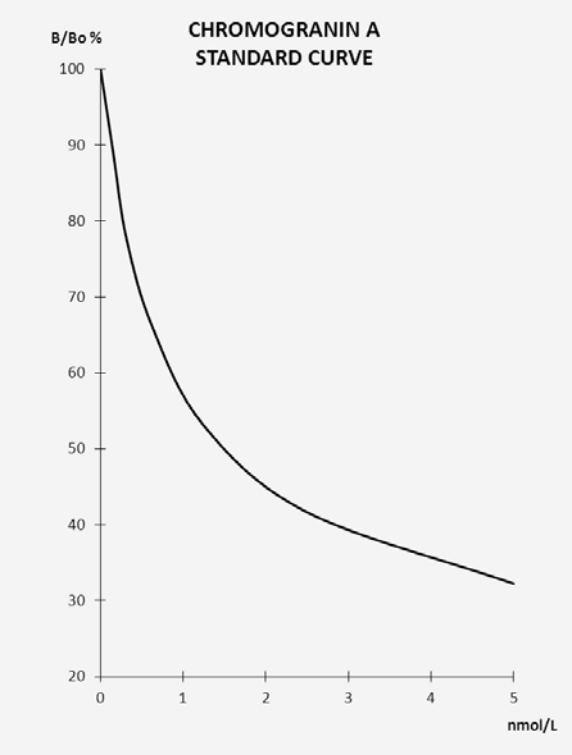
OUTLINE OF THE RIA PROCEDURE

Type of tubes	Tube no	Standard sample or control	^{125}I-Chromogranin A (B)	Anti-Chromogranin A (A)	Assay diluent (D)		Double antibody Solid phase (C)	
TOT	1- 2	-	100 µL	-		Vortex-mix and incubate for 20-24 hours at 2-8° C.	-	Vortex-mix and incubate for 30-60 min. at 2-8° C. Centrifuge 15 min. at 1700 x g at +4° C. Decant and count the radio-activity of the pellets.
NSB	3- 4	100 µL	100 µL	-	100 µL		500 µL	
Stand 0	5- 6	100 µL	100 µL	100 µL			500 µL	
Stand 0.16	7- 8	100 µL	100 µL	100 µL			500 µL	
Stand 0.32	9-10	100 µL	100 µL	100 µL			500 µL	
Stand 0.63	11-12	100 µL	100 µL	100 µL			500 µL	
Stand 1.25	13-14	100 µL	100 µL	100 µL			500 µL	
Stand 2.50	15-16	100 µL	100 µL	100 µL			500 µL	
Stand 5.00	17-18	100 µL	100 µL	100 µL			500 µL	
Control F	19-20	100 µL	100 µL	100 µL			500 µL	
Control G	21-22	100 µL	100 µL	100 µL			500 µL	
Sample 1	23-24	100 µL	100 µL	100 µL			500 µL	
Sample 2	25-26	100 µL	100 µL	100 µL			500 µL	
etc.			100 µL	100 µL				

EXAMPLE OF STANDARD CURVE

	Average cpm	Corrected cpm	B/T %	B/Bo %
Total counts	16079			
NSB	731			
Standard 0 nmol/L	6603	5872	37	100
Standard 0.156 nmol/L	5945	5214	32	89
Standard 0.313 nmol/L	5296	4565	28	78
Standard 0.625 nmol/L	4612	3882	24	66
Standard 1.25 nmol/L	3846	3115	19	53
Standard 2.50 nmol/L	3176	2446	15	42
Standard 5.00 nmol/L	2624	1893	12	32

Example of standard curve



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SYMBOLS USED ON LABELS

	Batch code.
	Catalogue number.
	Use by date.
	Temperature limit.
	Date of manufacture.
	Contains radioactive substances.
	Biological risks.
	Consult instructions for use.
	Manufacturer.
 **100**	Contains sufficient for 100 tests.

REAG A Ab	Anti-chromogranin A.
REAG B Ag ^{125}I	^{125}I-chromogranin A.
REAG C DASP	Double antibody solid phase.
REAG D DIL AS	Assay diluent.
REAG E CAL 10.0	Chromogranin A standard 10.0 nmol/L.
REAG F CONTROL	Control, level 1 (normal).
REAG G CONTROL	Control, level 2 (high).

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