Instruction

EURIA-Chromogranin B

Chromogranin B radioimmunoassay

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

REF **RB 322 RUO**

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PURPOSE OF RESEARCH PRODUCT

The EURIA-Chromogranin B kit is a competitive radioimunoassay (RIA) for determination of chromogranin B in human plasma and serum using antibodies against human chromoganin B. The result shall not be used for clinical diagnosis or patient management.

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 chromogranins. The neuroendocrine tumours are derived from the neuroendocrine cells and typical neuroendocrine tumours are carcinoid tumours, pheochromocytomas, neuroblastomas, small cell lung cancers, parathyroid adenomas, pituitary tumours, prostate cancers and pancreatic islet tumours and including the MEN1 and MEN2 syndromes. This also includes the different neuroendocrine tumour syndromes, such as gastrinomas, insulinomas, glucagonomas, somatostatinomas. PPomas and the non-functioning neuroendocrine tumours (Eriksson, B. et al. 2000, Stridsberg, M. et al. 1995, Stridsberg, M. et al. 2000). For all neuroendocrine tumours, chromogranin A has been shown to be the best circulating marker (Bajetta, E. et al. 1999).

Chromogranin A is an established marker for identification of neuroendocrine tumours. Despite its merits there are some drawbacks to chromogranin A measurements. It is known that chromogranin A levels are often elevated in individuals with decreased renal function, atrophic gastritis and in individuals being treated with proton pump inhibitor. These scenarios can sometimes influence assessment of chromogranin A. To circumvent this problem a test for chromogranin B has been developed. Chromogranin B has been shown to be a useful laboratory tool with similar applications as chromogranin A and it has been shown that Chromogranin B levels are not affected by either proton pump inhibitor treatment or decreased renal function (Stridsberg, M. et al. 2007). With its lower sensitivity but very high specificity chromgranin B measurements can serve as a valuable complement to chromogranin A. Euro-Diagnosticas Chromogranin B RIA is a competitive method based on polyclonal antibodies raised in rabbits. The antibodies were raised against a synthetic peptide containing amino acid sequence 439-451 in the chromogranin B molecule (Stridsberg, M. et al. 2005).

PRINCIPLE OF THE METHOD

The intended use of these reagents is the determination of chromogranin B in human serum or plasma. The basic principle for determination of chromogranin B with the Euro-Diagnostica chromogranin B RIA kit is a competitive radioimmunoassay using antibodies against human chromogranin B. Chromogranin B in standards and samples compete with ^{125}I-labelled chromogranin B in binding to the antibodies. The ^{125}I-chromogranin B binds to the antibodies in an inverse proportion to the concentration of chromogranin B in standards and samples. Antibody-bound ^{125}I-chromogranin B is separated from the unbound fraction using the double antibody solid phase technique. The bound fraction of ^{125}I-chromogranin B is measured in a gamma counter.

PHYSIOLOGICAL CONSIDERATIONS

Tumours with neuroendocrine differentiation often present with increased serum/plasma levels of chromogranin B. 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 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.

Chromogranin B Reference range, heparin plasma: < 1.8 nmol/L.
Chromogranin B Reference range, serum: < 2.1 nmol/L.

The range was set as the 97.5 percentile.
Reference material 120 apparently healthy blood donors: 60 males and 60 females.
Ages 21-65 years.

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

The test should not be relied upon as the sole basis of medical interpretation, but should be used in combination with physiological symptoms and the results of other available tests.

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 ^{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 B (Reagent A)
Rabbit antiserum to human chromogranin B (amino acids 439-451). The antiserum is diluted and lyophilized in 4.0 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 21.0 mL distilled water.

2. ^{125}I-Chromogranin B (Reagent B)
Activity: 56 KBq (1.5 μCi) on activity reference date. Lyophilized in 5.0 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 25 mL distilled water.

3. Double antibody solid phase (Reagent C)
Anti-rabbit-Ig coupled to cellulose particles in 0.01 M phosphate buffer pH 6.8 with 0.25% Human serum albumin, 0.045% NaCl, 0.05% NaN_3, 0.185% EDTA and 0.05% Tween 80.
11 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 B standard (Reagent E)
Concentration: 4.0 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 B.
Volume: 1.00 mL of each control after reconstitution. The chromogranin B concentrations are given on the labels of the vials. The controls should not be diluted after reconstitution.
Lyophilized in 1.00 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 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 10 weeks.

Reagent A: Anti-Chromogranin B
Reconstitute with 21.0 mL distilled water.
Store at 2-8° C.

Reagent B: ^{125}I-Chromogranin B
Reconstitute with 25 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 B 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

The assay is recommended for serum/heparin plasma (144 U.S.P. Heparin in a 10 mL tube).
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.
Avoid repeated freeze/thawing.
EDTA-containing tubes can NOT be used.

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.

A complete assay includes:

Standards (St-tubes): 7 different concentrations, 0, 0.125, 0.25, 0.50, 1.00, 2.00 and 4.00 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 1:5 with the assay diluent (Reagent D) before assay.
Samples with chromogranin B concentrations > 20 nmol/L (value 4 nmol/L by standard curve) should be reported as > 20 nmol/L or 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 B working standards by dilution of the chromogranin B standard 4.00 nmol/L (Reagent E) with assay diluent (Reagent D) according to the following example:
   a. Reagent E after reconstruction = 4.00 nmol/L
   b. 0.40 mL standard 4.00 nmol/L + 0.40 mL assay diluent = 2.00 nmol/L
   c. 0.40 mL standard 2.00 nmol/L + 0.40 mL assay diluent = 1.00 nmol/L
   d. 0.40 mL standard 1.00 nmol/L + 0.40 mL assay diluent = 0.50 nmol/L
   e. 0.40 mL standard 0.50 nmol/L + 0.40 mL assay diluent = 0.25 nmol/L
   f. 0.40 mL standard 0.25 nmol/L + 0.40 mL assay diluent = 0.125 nmol/L
   g. Assay diluent = 0 nmol/L
   Store the standards at -20° C or lower if reused.

3. **Dilute the samples 1:5** with assay diluent e.g. 100 µL sample and 400 µL assay diluent. Vortex-mix carefully.

4. Pipette 100 µL of standards (0-4.00 nmol/L) controls and samples in their respective tubes.

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

6. Pipette 200 µL anti-chromogranin B (Reagent A) in all tubes except the NSB-tubes and TOT-tubes.

7. Pipette 200 µL ^{125}I-chromogranin B (Reagent B) in all tubes. The TOT-tubes are sealed and kept aside.

8. Pipette 200 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 100 µL double antibody solid phase (Reagent C) in all tubes except the TOT-tubes. Vortex-mix carefully. Stir continuously during pipetting this reagent.

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

13. Centrifuge for 15 minutes at +4° C (minimum 1700 x g).
    Note: The correct centrifugation force is important for accurate performance.

14. Decant the supernatants.
    Note: The accurateness and coherency in handling of supernatants are crucial for the assay precision.

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 B standards.

3. Interpolate the chromogranin B concentrations of the controls and samples from the generated standard curve. Multiply the found concentrations in the samples with the dilution factor 5 (or actual dilution factors if further dilution has been done).

4. The standard curve and the calculation of the chromogranin B 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 parameters 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.
If any of the control values are not within their respective range, the test should be considered invalid and repeated.

2. Total counts
Counts obtained should approximate the expected CPM when adjusted for counter efficiency and radioactive decay. The content of ^{125}I-chromogranin B 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 40-64% at the activity reference date.

$\frac{Bo}{TOT} \times 100$ decreases 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
Monitor the ED80, ED50 and ED20 points of the standard curve for run to run reproducibility.

ASSAY CHARACTERISTICS

Sensitivity
The lowest detectable concentration in the assay is 0.039 nmol/L. This figure corresponds to a decrease in binding of 2 x SD of the bound radioactivity in the zero-concentration standard. Hence, the lowest detectable concentration for a sample diluted 1:5 will be 0.19 nmol/L.

Clinical sensitivity
43 heparin plasma samples from individuals with clinical diagnosis of NET (Neuroendocrine Tumours) were assayed.
The following table summarises the results.

Disease groups	Total number	Positive >1.8 nmol/L	Negative <1.8 nmol/L	Sensitivity %
FGC	8	5	3	63
EPT	11	10	1	91
MGC	21	17	4	81
FEO	2	2	0	100
NEC	1	0	1	0
	43	**34**	**9**	**79**

FGC = Foregut carcinoid
EPT = Endocrine pancreatic tumor
MGC = Midgut carcinoid
FEO = Pheochromocytoma
NEC = Neuroendocrine carcinoma

Specificity
The antiserum recognizes intact chromogranin B and fragments containing peptid sequence 439-451.

120 plasma/serum samples from apparently healthy blood donors were assayed with following results:

Specificity heparin plasma
= 118/120 = 98.3% 95% CI = 94.1-99.8%

Specificity serum
= 117/120 = 97.5% 95% CI = 92.9-99.5%

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

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

Precision
Intra assay variation:

Level	Coefficient of variation (%CV)	N
1.20 nmol/L	11.2%	30
6.65 nmol/L	3.8%	30

Inter assay variation (total variation):

Level	Coefficient of variation (%CV)	N
1.18 nmol/L	6.1%	10
6.24 nmol/L	3.7%	10

Linearity

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	1/5	4.66*	4.66	100
	1/10	2.31	2.33	99
	1/20	1.23	1.17	105
	1/40	0.61	0.58	105
	1/80	0.29	0.29	100
Sample	**Dilution**	**Mean Measured Concentration (nmol/L)**	**Calculated Concentration (nmol/L)**	**Dilution Corrected % Recovery**
2	1/5	4.10*	4.10	100
	1/10	2.30	2.05	112
	1/20	1.09	1.03	106
	1/40	0.59	0.51	116
	1/80	0.28	0.26	108
Sample	**Dilution**	**Mean Measured Concentration (nmol/L)**	**Calculated Concentration (nmol/L)**	**Dilution Corrected % Recovery**
3	1/5	4.61*	4.61	100
	1/10	2.56	2.31	111
	1/20	1.21	1.15	105
	1/40	0.60	0.58	103
	1/80	0.32	0.29	110

*NB. This point was intentionally chosen to demonstrate dilution linearity in samples with extrapolated levels moderately higher than the range of the standard curve.

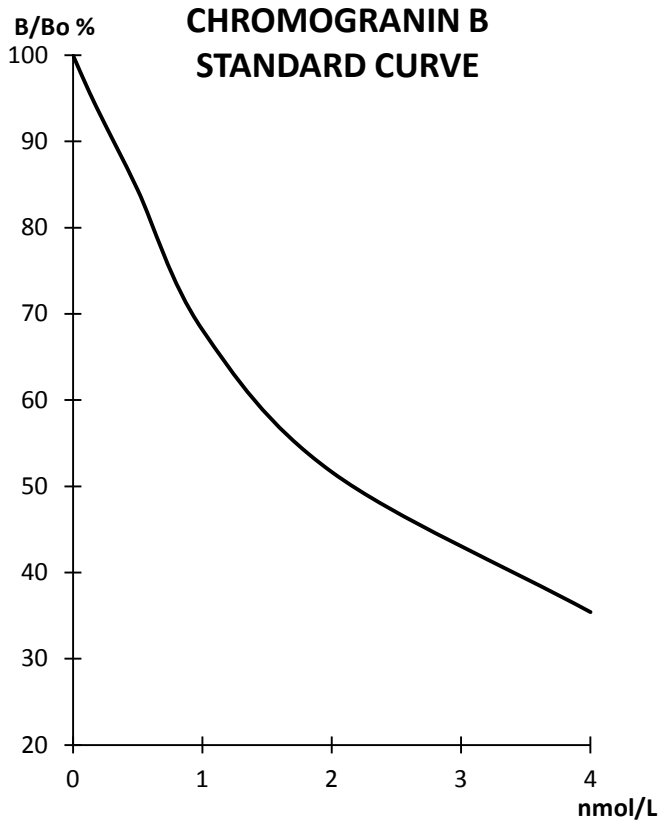
OUTLINE OF THE RIA PROCEDURE

Type of tubes	Tube no	Standard sample or control	Anti-Chromogranin B (A)	^{125}I-Chromogranin B (B)	Assay diluent (D)		Double antibody Solid phase (C)	
TOT	1-2	-	-	200 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 radioactivity of the pellets.
NSB	3-4	100 µL	-	200 µL	200 µL		100 µL	
Stand 0	5-6	100 µL	200 µL	200 µL			100 µL	
Stand 0.12	7-8	100 µL	200 µL	200 µL			100 µL	
Stand 0.25	9-10	100 µL	200 µL	200 µL			100 µL	
Stand 0.50	11-12	100 µL	200 µL	200 µL			100 µL	
Stand 1.00	13-14	100 µL	200 µL	200 µL			100 µL	
Stand 2.00	15-16	100 µL	200 µL	200 µL			100 µL	
Stand 4.00	17-18	100 µL	200 µL	200 µL			100 µL	
Control F	19-20	100 µL	200 µL	200 µL			100 µL	
Control G	21-22	100 µL	200 µL	200 µL			100 µL	
Sample 1	23-24	100 µL	200 µL	200 µL			100 µL	
Sample 2	25-26	100 µL	200 µL	200 µL			100 µL	
etc.			200 µL	200 µL			100 µL	

EXAMPLE OF STANDARD CURVE

	Average cpm	Corrected cpm	B/T %	B/Bo %
Total counts	20974			
NSB	1232			
Standard 0 nmol/L	10248	9016	43	100
Standard 0.125 nmol/L	9866	8634	41	96
Standard 0.25 nmol/L	9518	8286	40	92
Standard 0.50 nmol/L	8839	7607	36	84
Standard 1.00 nmol/L	7377	6145	29	68
Standard 2.00 nmol/L	5890	4658	22	52
Standard 4.00 nmol/L	4425	3193	15	35

EXAMPLE OF CHROMOGRANIN B STANDARD CURVE



Note: The curve is an example and should not be used for interpretation.

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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 B.
REAG B Ag ^{125}I	^{125}I-chromogranin B.
REAG C DASP	Double antibody solid phase.
REAG D DIL AS	Assay diluent.
REAG E CAL 4.0	Chromogranin B standard 4.0 nmol/L.
REAG F CONTROL	Control, level 1 (normal).
REAG G CONTROL	Control, level 2 (high).

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