

Instruction
EURIA-NPY

Neuropeptide Y radioimmunoassay

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REF

RB 317 RUO



100

INTRODUCTION

Neuropeptide Y (NPY) is a 36 aminoacid residues peptide which was initially isolated from pig brain (1,2). NPY was later extracted from human adrenal-medullary phaeochromocytoma tissue. The human NPY differs from the pig NPY only by the replacement of leucine at position 17 by methionine (3). NPY shares considerable sequence homology with pancreatic polypeptide and peptide YY (4). NPY-like immunoreactivity has been found in the central nervous system and in peripheral noradrenergic neurons and intestinal neurons (5,6,7,8). Potent vasoconstrictor activity is exhibited by NPY (9). NPY also inhibits noradrenaline release via a presynaptic action (10) and has stimulatory effects on the contraction of the heart (11). Phaeochromocytoma tumours (3,12,13) contain NPY and elevated levels of NPY in plasma from phaeochromocytoma patients have been reported (14). Increased plasma concentrations of NPY have been found in patient with neuroblastoma (15). Increased plasma concentrations of NPY have also been found in pediatric B-cell precursor leukemia (16).

PRINCIPLE OF THE METHOD

The intended use of these reagents is for assay of NPY in human serum/plasma by direct assay without extraction. NPY in the serum/plasma samples is assayed by a competitive radioimmunoassay using an antiserum raised against synthetic NPY conjugated to bovine thyroglobulin. NPY in standards and samples compete with ¹²⁵I-labelled NPY in binding to the antibodies. ¹²⁵I-NPY binds in a reverse proportion to the concentration of NPY in standards and samples. Antibody-bound ¹²⁵I-NPY is separated from the unbound fraction by using double antibody coupled to solid phase. The radioactivity of the antibody-bound ¹²⁵I-NPY is measured. The antiserum used in this method crossreacts less than 2.0% with human Peptide YY. The result shall not be used for clinical diagnosis or patient management.

PHYSIOLOGICAL CONSIDERATIONS

Increased serum/plasma concentrations of NPY have been found in subjects with neuroblastoma (15). Increased serum/plasma concentrations of NPY have also been found in pediatric B-cell precursor leukemia (16).

Elevated levels of NPY have been found in serum/plasma from subjects with phaeochromocytoma tumours (3,12,13). Assay of NPY in serum/plasma may serve as valuable tool in the identification of neuroblastoma and pediatric B-cell precursor leukemia and phaeochromocytoma.

Normal level of NPY in human plasma

Subjects	Number	Range	Mean
Healthy normals (age 20-60 years)	109	36-120 pmol/L	74 pmol/L (SD= ± 15)

Normal level obtained in healthy people in the age 20-60 years.

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 are 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 drain pipes 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 each kit are sufficient for 100 tubes.

1. Anti-NPY (Reagent A)

Rabbit antiserum raised against NPY conjugated to bovine thyroglobulin. Lyophilized in 2.0 mL 0.5 M phosphate buffer, pH 7.4, with 2.5% human serum albumin, 2.5% EDTA, 1.0% Triton X-100, 0.5% sodium azide and 5000 KIU aprotinin (Trasylol® or equivalent) per mL. For 100 tubes. Reconstitution in 22 mL distilled water.
Colour: Yellow.

2. ¹²⁵I-NPY (Reagent B)

Contains 28 KBq or 0.75 µCi at reference date. Synthetic, human NPY is iodinated according to Bolton-Hunter. The monoiodinated form is purified by HPLC. Specific activity: 1700-2100 µCi/nmol (62-77 MBq/nmol). Lyophilized in 2.5 mL 0.5 M phosphate buffer, pH 7.4, with 2.5% human serum albumin, 2.5% EDTA, 1.0% Tween 80, 0.5% sodium azide and 5000 KIU aprotinin (Trasylol® or equivalent) per mL. Reconstitution in 25 mL distilled water.
Colour: Blue.

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% NaN₃, 0.185% EDTA and 0.05% Tween 80.
11.0 mL suspension.

4. Standard diluent (Reagent D)

10.0 mL NPY-free human serum, lyophilized. Contains 500 KIU aprotinin (Trasylol® or equivalent) per mL. Reconstitution in 10.0 mL distilled water. For preparation of NPY working standards.

5. NPY standard 3000 pmol/L (Reagent E)

Lyophilized. 2.00 mL standard. Concentration: 3000 pmol/L after reconstitution. The standard is produced from synthetic, human NPY. Lyophilized in 0.05 M phosphate buffer, pH 7.4, with 0.25% human serum albumin, 0.25% EDTA, 0.1% Triton X-100, 0.05% sodium azide and 500 KIU aprotinin (Trasylol® or equivalent) per mL. For preparation of NPY working standards.

6. Assay buffer (Reagent F)

5.0 mL 0.05 M phosphate buffer, pH 7.4, with 0.25% human serum albumin, 0.25% EDTA, 0.1% Triton X-100, 0.05% sodium azide and 500 KIU aprotinin (Trasylol® or equivalent) per mL.
To be used instead of antiserum in non-specific binding test tubes.

7. Controls (Reagent G-H)

Lyophilized serum controls with low and high concentration of NPY. 2.00 mL of each control after reconstitution. The NPY concentrations are given on the labels of the vials.
Contains 0.05% sodium azide.

REAGENT PREPARATION AND STORAGE

Store all reagents at 2-8° C before reconstitution and use. The water used for reconstitution of lyophilized reagents should be distilled in an all-glass apparatus or be of corresponding purity. Dissolve the contents in a vial by gentle inversion and avoid foaming. The stability of the reagents is found on the labels of the vials. For lyophilized reagents the expiry dates are valid for the unreconstituted reagents. The reconstituted reagents are stable for 10 weeks (no longer than to the expiry date) if stored properly.

Reagent A: Anti-NPY

Reconstitute with 22 mL distilled water.
Store at 2-8° C.

Reagent B: ¹²⁵I-NPY

Reconstitute with 25 mL distilled water.
Store at -18° C or lower if reused.

Reagent C: Double antibody solid phase

Ready for use. Stir continuously during pipetting of this reagent.
Store at 2-8° C.

Reagent D: Standard diluent

Reconstitute with 10.0 mL distilled water.
Store at -18° C or lower if reused.

Reagent E: NPY standard, 3000 pmol/L

Reconstitute with 2.00 mL distilled water.
Store at -18° C or lower if reused.
For preparation of NPY-working standards, see radioimmunoassay procedure.

Reagent F: Assay buffer

Ready for use.
Store at 2-8° C.

Reagent G-H: Controls

Reconstitute with 2.00 mL distilled water. Store at -18° C or lower if reused.

EQUIPMENT AND REAGENTS REQUIRED BUT NOT PROVIDED

Disposable test tubes 11-13 x 55 mm, polystyrene

Pipettes with disposable tips, 100 and 200 μ L

A repeating pipette, e.g. Eppendorf Multipipette, for volumes 100 and 200 μ L will facilitate the dispensing of the reagents

Variable pipette 200-1000 μ L

Vortex mixer

Centrifuge capable for min 1700 x g (refrigerated centrifuge is preferred)

Gammacounter

Distilled water

SPECIMEN COLLECTION

Vein blood is collected in tubes without additives. The sample is allowed to clot. The serum is separated by centrifugation at +4°C. The serum should be frozen at -20°C within 1 hour. The serum should be stored at -20°C or lower until assayed.

Plasma samples (EDTA or Heparin) can also be used but should not be diluted (dilution of plasma with the standard diluent may lead to clotting in the assay during incubation).

Repeated freezing and thawing should be avoided.

RADIOIMMUNOASSAY PROCEDURE

Reconstitute the reagents as specified. 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, 9.4, 18.8, 37.5, 75, 150 and 300 pmol/L.

Controls (C-tubes): Controls with known concentrations of NPY.

Samples (P-tubes).

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

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

For an overview see table 1 on page 11.

PERFORMANCE

1. Reconstitute the reagents according to the instructions.
2. Prepare the NPY-working standards by dilution of the NPY-standard 3000 pmol/L (Reagent E) with the standard diluent (Reagent D) according to the following:
A/ 0.200 mL standard 3000 pmol/L + 1.800 mL diluent = 300 pmol/L
B/ 1.00 mL standard 300 pmol/L + 1.00 mL diluent = 150 pmol/L
C/ 1.00 mL standard 150 pmol/L + 1.00 mL diluent = 75 pmol/L
D/ 1.00 mL standard 75 pmol/L + 1.00 mL diluent = 37.5 pmol/L
E/ 1.00 mL standard 37.5 pmol/L + 1.00 mL diluent = 18.8 pmol/L
F/ 1.00 mL standard 18.8 pmol/L + 1.00 mL diluent = 9.4 pmol/L
G/ Standard diluent = 0 pmol/L.
Store the standard solutions at -18° C or lower if reused.
3. Pipette 200 µL of the standards (0-300 pmol/L), samples and controls in their respective tubes. Pipette 200 µL of the zero-standard in the NSB-tubes.
4. Pipette 200 µL anti-NPY (Reagent A) to all tubes except the NSB- and TOT-tubes.
5. Add 200 µL assay buffer (Reagent F) to the NSB-tubes.
6. Vortex-mix and incubate for 20-24 hours at 2-8° C.
7. Pipette 200 µL ¹²⁵I-NPY (Reagent B) to all tubes. The TOT-tubes are sealed and kept aside.
8. Vortex-mix and incubate for 20-24 hours at 2-8° C.
9. Pipette 100 µL double antibody-solid phase (Reagent C) to all tubes except the TOT-tubes. (stir continuously during pipetting).
10. Vortex-mix carefully and incubate for 30-60 minutes at 2-8° C.
11. Centrifuge the tubes for 15 minutes at +4° C (minimum 1700 x g).
12. Decant the supernatants immediately after centrifugation.
13. Count the radioactivity of the precipitates in a gamma counter (counting time: 2-4 minutes).

CALCULATION OF RESULTS

1. Subtract the average count rate (CPM) of the non-specific binding tubes from the count rates (CPM) of the replicates of standards, controls and samples.
2. A standard curve is generated by plotting the precipitated CPM, bound fraction in CPM or % B/TOT against the concentrations of the NPY-standards. An example of a standard curve is given on page 12.
3. Interpolate the NPY concentrations of the samples and controls from the generated standard curve.
4. The standard curve and the calculations of the concentrations in samples and controls can also be done by a computer method.

DILUTION OF SAMPLES

Serum samples with NPY concentrations above 300 pmol/L should be diluted with standard diluent (reagent D) for determination of the real concentration of NPY.

The following procedure is recommended:

Dilution 1:2: Pipette 100 μ L sample and 100 μ L standard diluent in the assay tube (perform in duplicate).

Dilution 1:4: Pipette 50 μ L sample and 150 μ L standard diluent in the assay tube (perform in duplicate).

ASSAY CHARACTERISTICS

Sensitivity

The lowest detectable concentration is 3 pmol/L. The figure corresponds to a decrease in binding of 2 x SD of the bound radioactivity in the zero-concentration standard.

Accuracy

A mean recovery of 82.4% (range 75-88%) was achieved in the assay of plasma samples with known amounts of NPY added. NPY was added in the range: 50-150 pmol/L.

Precision

Intra assay variation

<u>Level</u>	<u>Coefficient of variation (%CV)</u>	<u>N</u>
43.7 pmol/L	5.0	8
98.9 pmol/L	4.5	8

Inter assay variation (total variation)

<u>Level</u>	<u>Coefficient of variation (%CV)</u>	<u>N</u>
42.4 pmol/L	9.2	6
90.2 pmol/L	7.6	6

Specificity

The following cross-reactions with related peptides have been found:

Compound	Cross-reaction
Neuropeptide Y, human	100.0%
Peptide YY, human	<0.1%
Pancreatic Polypeptide, human	<0.1%
PYY 3-36	<0.1%
NPY 22-36	<0.1%
PP Bovine	<0.1%

Interference

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

QUALITY CONTROL

In order for the laboratory to completely monitor the consistent performance of the radioimmunoassay there are some important factors which must be checked.

1. Controls

The found concentrations of the controls (reagent G and H) should be within the limits given on the labels of the vials.

2. Total counts (TOT)

Counts obtained should approximate the expected CPM when adjusted for counter efficiency and radioactive decay. The content of ¹²⁵I-NPY in this kit will give 10 500 CPM (-5% to +20%) at the activity reference date (counter efficiency = 80%).

3. Maximum binding (Bo/TOT)

Calculate for each assay the % bound radioactivity in the zero-standard:

$$\frac{B_0}{TOT} \times 100$$

4. Non-specific binding (NSB/TOT)

Calculate for each assay the % non-specific binding $\frac{NSB}{TOT} \times 100$

$$\frac{NSB}{TOT} \times 100 \text{ is less than } 8\%.$$

5. Shape of standard curve

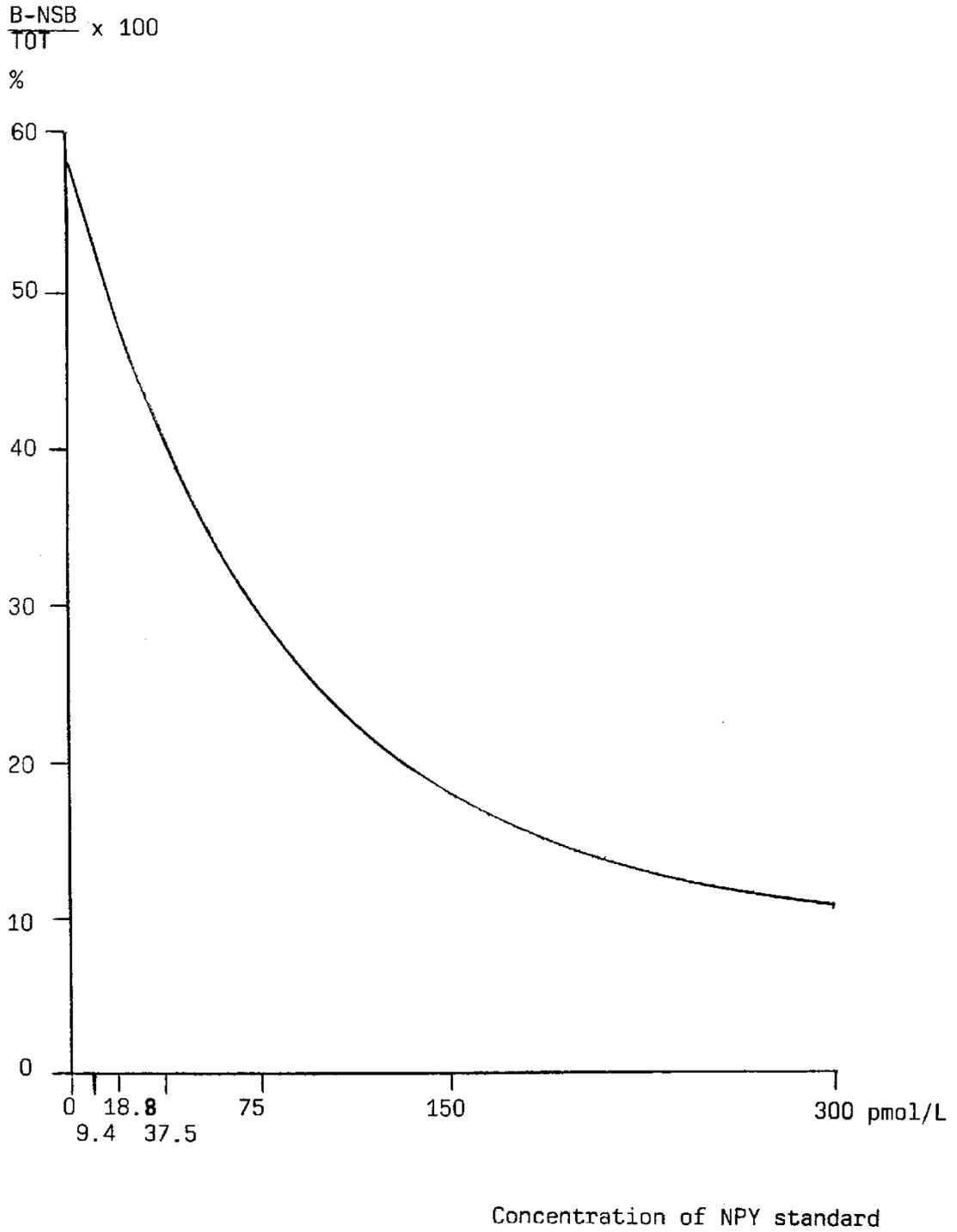
For example, monitor the 80, 50 and 20% points of the standard line for run to run reproducibility.

OUTLINE OF THE RIA PROCEDURE

Type of tubes	Tube no	Standard sample or control	Anti-NPY (A)	Assay buffer (F)		¹²⁵ I-NPY (B)		Double antibody solid phase (C)	
TOT	1-2	-	-	-	Vortex-	200 µL	Vortex-	-	Vortex-mix
NSB	3-4	200 µL	-	200	mix and	200 µL	mix and	100 µL	and
Stand 0	5-6	200 µL	200 µL	-	incubate	200 µL	incubate	100 µL	incubate
Stand 9.4	7-8	200 µL	200 µL	-	for 20-24	200 µL	for 20-24	100 µL	for 30-60
Stand 18.8	9-10	200 µL	200 µL	-	hours at	200 µL	hours at	100 µL	min. at
Stand 37.5	11-12	200 µL	200 µL	-	2-8° C.	200 µL	2-8° C.	100 µL	2-8° C.
Stand 75	13-14	200 µL	200 µL	-		200 µL		100 µL	Centrifuge
Stand 150	15-16	200 µL	200 µL	-		200 µL		100 µL	15 min. at
Stand 300	17-18	200 µL	200 µL	-		200 µL		100 µL	1700 x g at
Control (G)	19-20	200 µL	200 µL	-		200 µL		100 µL	+4° C.
Control (H)	21-22	200 µL	200 µL	-		200 µL		100 µL	Decant and
Sample 1	23-24	200 µL	200 µL	-		200 µL		100 µL	count the
Sample 2	25-26	200 µL	200 µL	-		200 µL		100 µL	radioactivi- ty of the precipitates.

Table 1

EXAMPLE OF NPY STANDARD CURVE



REFERENCES

1. Tatemoto, K.
Neuropeptide Y: Complete amino acid sequence of the brain peptide.
Proc Natl Acad Sci USA 79:5485, 1982
2. Tatemoto, K., Carlquist, M. and Mutt, V.
Neuropeptide Y - a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptides.
Nature (London) 296: 659, 1982.
3. Corder, R., Emson, P.C. and Lowry, P.J.
Purification and characterization of human neuropeptide Y from adrenal medullary pheochromocytoma tissue.
Biochem J 219:699, 1984.
4. Tatemoto, K.
Isolation and characterization of peptide YY (PYY) a candidate gut hormone that inhibits pancreatic exocrine secretion.
Proc Natl Acad Sci USA 79:2514, 1982.
5. Lundberg, J.M., Terenius, L., Hökfelt, T. et al.
Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function.
Acta Physiol Scand 116:477, 1982.
6. Allen, Y.S., Adrian, T.E., Allen, J.M. et al.
Neuropeptide Y distribution in rat brain.
Science 221:877, 1983.
7. Lundberg, J.M., Terenius, L., Hökfelt T. and Goldstein, M.
High levels of neuropeptide Y (NPY) in peripheral noradrenergic neurons in various mammals including man.
Neurosci Lett 42:167, 1983.
8. Furness, J.B., Costa, M., Emson, P.C. et al.
Distribution, pathways and reactions to drug treatment of nerves with neuropeptide Y and pancreatic polypeptide-like immunoreactivity in the guinea pig digestive tract.
Cell Tissue Res 234:71, 1983.
9. Lundberg, J.M. and Tatemoto, K.
Pancreatic polypeptide family (APP, BPP, NPY and PYY) in relation to sympathetic vasoconstriction resistant to α -adrenoceptor blockade.
Acta Physiol Scand 116:393, 1982.
10. Lundberg, J.M. and Stjärne, L.
Neuropeptide Y (NPY) depresses the secretion of 3H-noradrenaline and contractile response evoked by fieldstimulation in rat vas deference.
Acta Physiol Scand 120:477, 1984.

11. Lundberg, J.M., Hua, X.Y. and Franco-Cereceda, A.
Effects of neuropeptide Y (NPY) on mechanical activity and neurotransmission in the heart, vas deferens and urinary bladder of the guinea-pig.
Acta Physiol Scand 121:325, 1984.
12. Adrian, T.E., Terenghi, G., Brown, M.J. et al.
Neuropeptide Y in pheochromocytomas and ganglioneuroblastomas.
Lancet ii:540, 1983.
13. Emson, P.C., Corder, R. and Lowry, P.J.
Demonstration of neuropeptide Y-like immunoreactivity in human pheochromocytoma extracts.
Regulatory Peptides 8:89, 1984.
14. Theodorsson-Norheim, E., Hemsen, A. and Lundberg, J.M.
Radioimmunoassay for neuropeptide Y (NPY): Chromatographic characterization of immunoreactivity in plasma and tissue extracts.
Scand J Clin Lab Invest 45:355, 1985.
15. Kogner, P., Björk, O. and Theodorsson, E.
Neuropeptide Y in neuroblastoma: Increased concentration in metastasis, release during surgery, and characterization of plasma and tumour extracts.
Medical and Pediatric Oncology 21:317-322, 1993.
16. Kogner, P., Ericsson, A., Barbany, G., Persson, H., Theodorsson, E. and Björk, O.
Neuropeptide Y (NPY) synthesis in lymphoblasts and increased plasma NPY in pediatric B-cell precursor leukemia.
Blood, vol. 80, no. 5 (september 1), 1992:pp 1324-1329.
17. Kalra, S.P., Gube, M.G., Bin Xu, S.P., Horvath, T.L. and Kalra P.S.
Interacting Appetite-Regulating Pathways in the Hypothalamic Regulation of Body Weight.
Endocrine reviews 20(1):68-100, (1999).
18. Kallio, J., Pesonen, U., Kaipio, K., Karvonen, M.K., Jaakkola, U, Heinonen, O.J., Uusitupa, M.I.J. and Koulu, M.
Altered intracellular processing and release of neuropeptide Y due to leucine 7 to proline 7 polymorphism in the signal peptide of preproneuropeptide Y in humans.
The FASEB Journal express article 10.1096/fj. 00-0437 fje. Published online March 12, 2001.

SYMBOLS USED ON LABELS

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

REAG A Ab	Anti-NPY
REAG B Ag ¹²⁵I	¹²⁵ I-NPY.
REAG C DASP	Double antibody solid phase.
REAG D DIL CAL	Standard diluent.
REAG E CAL 3000	NPY standard 3000 pmol/L.
REAG F BUF AS	Assay buffer.
REAG G CONTROL	Control, level 1 (low).
REAG H CONTROL	Control, level 2 (high).

EURO DIAGNOSTICA AB

Lundavägen 151, SE-212 24 Malmö, Sweden
 Phone: +46 40 53 76 00, Fax: +46 40 43 22 88
 E-mail: info@eurodiagnostica.com
www.eurodiagnostica.com