Human NPY
96-Well Plate Assay
Cat. # EZHNPY-25K

HUMAN NPY ELISA KIT
96-Well Plate (Cat. # EZHNPY-25K)

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HUMAN NPY ELISA Kit
96-Well Plate (Cat.# EZHNPY-25K)

I. INTENDED USE

This kit is used for the non-radioactive quantification of Neuropeptide Y (NPY) in human serum and plasma. One kit is sufficient to measure 38 unknown samples in duplicate. ***This kit is for research purpose only.***

II. PRINCIPLES OF ASSAY

This assay is a Sandwich ELISA based on: 1) capture of NPY in the sample by anti-human NPY IgG and immobilization of the resulting complex to the wells of a microtiter plate coated by a pre-titered amount of anchor antibodies, 2) binding of a second biotinylated antibody to NPY after brief washings, 3) wash away of unbound materials, followed by conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away of free enzyme, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetra-methylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured NPY in the unknown sample, the concentration of NPY can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of Human NPY.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate and contains the following reagents:

1. Microtiter Plate

Coated with pre-titered anchor antibodies.

Quantity: 1 Strip Plate

Preparation: Ready to use.

Note: Unused strips should be resealed in the foil pouch with the dessicant provided and stored at 2-8 ℃.

2. Adhesive Plate Sealer

Quantity: 2 sheets

Preparation: Ready to use.

3. 10X HRP Wash Buffer Concentrate

10X concentrate of 50mM Tris Buffered Saline containing Tween-20.

Quantity: 2 bottles containing 50ml each

Preparation: Dilute 1:10 with distilled or de-ionized water.

4. Human NPY Standard

Human NPY reference standard, 1ng/ml, lyophilized

Quantity: 1 bottle, 1ng/ml after reconstitution with appropriate amount of water.

Preparation: Hydrate thoroughly in distilled or de-ionized water immediately before use. Please refer to the analysis sheet for exact amount of water to be used since it will be lot dependent.

5. Human NPY Quality Controls 1 and 2

One vial each, lyophilized, containing human NPY at two different levels.

Quantity: 0.5 ml/vial upon hydration.

Preparation: Reconstitute each vial with 0.5 ml de-ionized water immediately before use.

6. Matrix Solution

Processed serum matrix containing 0.08% Sodium Azide

Quantity: 1 ml/vial

Preparation: Ready to use.

III. REAGENTS SUPPLIED

7. Assay Buffer

0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide, and 1% BSA.

Quantity: 20 ml/vial

Preparation: Ready to use.

8. Human NPY Capture Antibody

Pre-titered capture antibody solution in buffer

Quantity: 3 ml/vial

Preparation: Ready to use.

9. Human NPY Detection Antibody

Pre-titered detection antibody solution in buffer

Quantity: 12 ml/vial

Preparation: Ready to use.

10. Enzyme Solution

Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.

Quantity: 12 ml/vial

Preparation: Ready to use.

11. Substrate

3, 3',5,5'-tetramethylbenzidine in buffer.

Quantity: 12 ml/vial

Preparation: Ready to use. Minimize the exposure to light.

12. Stop Solution

0.3 M HCl

Quantity: 12 ml/vial

Preparation: Ready to use.

[Caution: Corrosive Solution]

IV. STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2-8°C. For longer storage (> 2 weeks), freeze diluted Wash Buffer, Assay Buffer, Matrix Solution and reconstituted Standards and Controls at ≤ –20°C. Minimize repeated freeze and thaw of the Standards and Quality Controls. Unused microtiter strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTIONS

1. Sodium Azide

Sodium azide has been added to certain 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 pluming to form explosive metal azides. On disposal, flush with large volume of water to prevent azide build up.

2. Hydrochloric Acid

Hydrochloric acid is corrosive, can cause eye and skin burns. Harmful if swallowed. Causes respiratory and digestive tract burns. Avoid contact with skin and eye. Do not swallow or ingest.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

Pipettes and pipette tips: 10µl ~ 20 µl or 20µl ~ 100 µl

1. Multi-channel Pipettes and pipette tips: 5 ~ 50 µl and 50 ~ 300 µl
2. Buffer and Reagent Reservoirs
3. Vortex Mixer
4. De-ionized Water
5. Microtiter Plate Reader capable of reading absorbency at 450 nm and 590nm
6. Orbital Microtiter Plate Shaker
7. Absorbent Paper or Cloth
8. Aprotinin is recommended for Sample Collection and Storage.
9. DPP-IV Inhibitor (we recommend Millipore Cat# DPP4-010) is required for Sample Collection and Storage.

VII. SAMPLE COLLECTION AND STORAGE

NPY (3~36) amide and smaller fragments have been reported to be present in human serum/plasma. For best results, we recommend NPY protection from proteolytic degradation by treating blood samples with Millipore DPP-IV Inhibitor (Cat# DPP4-010) or and Aprotinin immediately after the blood is drawn.

1. To prepare serum, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Immediately add enough DPP-IV Inhibitor and Aprotinin to a final concentration of 50 µM and 500 KIU/ml, respectively. Let blood clot at room temperature for 30 min.

2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2°C.

3. Transfer and aliquot serum samples in separate tubes of small quantity. Date and identify each sample.

4. Use freshly prepared serum or store samples at –20 ± 5°C for later use. Avoid multiple (> 5) freeze/thaw cycles.

5. To prepare plasma sample, whole blood should be collected into a centrifuge tube containing enough K_3 EDTA to achieve a final concentration of 1.735 mg/ml and treated with DPP-IV Inhibitor and Aprotinin as described for serum, followed by immediate centrifugation. Observe same precautions in the preparation of serum samples.

6. If heparin is to be used as anti-coagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.

7. Avoid using samples with gross hemolysis or lipemia.

VIII. REAGENT PREPARATION

A. Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human NPY Standard with the amount of distilled or de-ionized water specified in the data sheet supplied with this kit to give a final concentration of 1 ng/ml (or 1,000 pg/ml) of NPY Standard. Invert and mix gently until completely in solution.

2. Label six tubes with the additional concentrations of standards to be prepared: 5 pg/ml, 10 pg/ml, 40 pg/ml, 100 pg/ml, 250 pg/ml and 500 pg/ml. Add Assay Buffer to each of the six tubes according to the volumes outlined in the chart below. Dilute the reconstituted 1ng/ml standard stock according to the chart below. Vortex each tube briefly to ensure complete mixing.

   Note: Change tip for every dilution. Wet tip with standard before dispensing. Unused portions of standard should be stored in small aliquots at ≤ -20°C. Avoid multiple freeze/thaw cycles.

Concentration of Standards	Volume of 1 ng/ml Stock to Add	Volume of Assay Buffer to Add
5 pg/ml	0.005 ml	0.995 ml
10 pg/ml	0.010 ml	0.990 ml
40 pg/ml	0.040 ml	0.960 ml
100 pg/ml	0.100 ml	0.900 ml
250 pg/ml	0.250 ml	0.750 ml
500 pg/ml	0.500 ml	0.500 ml
1,000 pg/ml	----	----

B. Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each Human NPY Quality Control 1 and Quality Control 2 with 0.5 ml distilled or de-ionized water and gently invert to ensure complete hydration. Unused portions of the reconstituted Quality Controls should be stored in small aliquots at ≤ -20°C. Avoid further freeze/thaw cycles.

IX. HUMAN NPY ELISA ASSAY PROCEDURE

Pre-warm all reagents to room temperature immediately before setting up the assay.

1. Dilute the 10X concentrated HRP wash buffer 10 fold by mixing the entire contents of both buffer bottles with 900 ml de-ionized or glass distilled water.

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and fill each well with 300 µl diluted Wash Buffer. Decant wash buffer and remove the residual amount by inverting the plate and tapping it smartly onto absorbent towels several times. Wash assay plate using this procedure 2 additional times. **Do not let wells dry before proceeding to the next step.** If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.

3. Add 50 µl Matrix Solution to Blank, Standards and Quality Control wells (refer to § X. for suggested well orientations).

4. Add 50 µl assay buffer to each of the Blank and sample wells.

5. Add in duplicate 50 µl NPY Standards in the order of ascending concentrations to the appropriate wells.

6. Add in duplicate 50 µl QC1 and 50 µl QC2 to the appropriate wells.

7. Add sequentially 50 µl of the unknown samples in duplicate to the remaining wells**.**

8. Add 20 µl Capture Antibody Solution to each well with a multi-channel pipette.

9. Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.

10. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.

11. Wash wells 3 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.

IX. HUMAN NPY ELISA ASSAY PROCEDURE (continued)

12. Add 100 µl Detection Antibody Solution to each well. Re-cover plate with sealer and incubate with moderate shaking at room temperature for 1 hour on the micro-titer plate shaker.

13. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well. Wash wells 3 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.

14. Add 100 µl Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 min on the micro-titer plate shaker.

15. Remove sealer, decant solutions from the plate and tap plate to remove the residual fluid.

16. Wash wells 6 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.

17. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for **approximately** 10 to 20 minutes. Blue color should be formed in wells of the NPY standards with intensity proportional to increasing concentrations of NPY.

    Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

18. Remove sealer and add 100 µL Stop Solution **[CAUTION: CORROSIVE SOLUTION]** and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Read absorbance at 450 nm and 590nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units.

Assay Procedure for Human NPY ELISA Kit (Cat. # EZHNPY-25K)

	Step 1	Step 2	Step 3	Step 4	Step 5-7	Step 8	Step 9-11	Step 12	Step 13	Step 14	Step 15-16	Step 17			
Well #	Dilute both bottles of 10X HRP Wash Buffer with 900 ml de-ionized water.	Wash plate 3X with 300 µl diluted HRP wash buffer. Remove residual buffer by tapping smartly on absorbent towels	Matrix Solution	Assay Buffer	Standards/ QCs/ Samples	Capture Antibody Solution	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µl Wash Buffer.	Detection Antibody Solution	Seal, Agitate, Incubate 1 hour at Room Temperature. Wash 3X with 300 µl Wash Buffer.	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 6X with 300 µl Wash Buffer.	Substrate	Seal, Agitate, Incubate 10~20 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1			50 µl	50 µl	--	20 µl		100 µl		100 µl		100 µl		100 µl	
C1, D1			50 µl	--	50 µl of 5 pg/ml Standard										
E1, F1			50 µl	--	50 µl of 10 pg/ml Standard										
G1, H1			50 µl	--	50 µl of 40 pg/ml Standard										
A2, B2			50 µl	--	50 µl of 100 pg/ml Standard										
C2, D2			50 µl	--	50 µl of 250 pg/ml Standard										
E2, F2			50 µl	--	50 µl of 500 pg/ml Standard										
G2, H2			50 µl	--	50 µl of 1,000 pg/ml Standard										
A3, B3			50 µl	--	50 µl of QC 1										
C3, D3			50 µl	--	50 µl of QC 2										
E3, F3			--	50 µl	50 µl of Sample 1										
G3, H3 Etc.			--	50 µl	50 µl of sample 2										

X. MICROTITER PLATE ARRANGEMENT

Human NPY ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	100 pg/ml	QC1	Etc.								
B	Blank	100 pg/ml	QC1	Etc.								
C	5 pg/ml	250 pg/ml	QC2									
D	5 pg/ml	250 pg/ml	QC2									
E	10 pg/ml	500 pg/ml	Sample 1									
F	10 pg/ml	500 pg/ml	Sample 1									
G	40 pg/ml	1000 pg/ml	Sample 2									
H	40 pg/ml	1000 pg/ml	Sample 2									

XI. CALCULATIONS

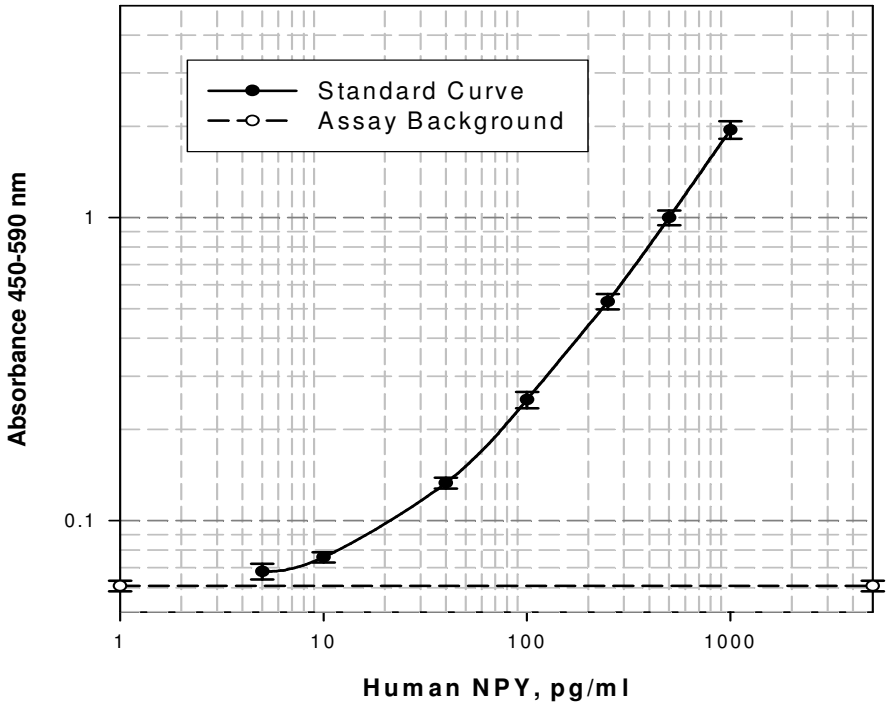
The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function

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). When sample volume assayed is less than 50 µl, compensate the volume deficit with matrix solution.

XII. INTERPRETATION

1. The assay will be considered accepted when all Quality Control values fall within the calculated QC range. If any QCs fall outside of the control range, review results with a supervisor.
2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.
3. The limit of sensitivity of this assay is 3 pg/ml NPY (50 µl sample size).
4. The appropriate range of this assay is 5 pg/ml to 1,000 pg/ml NPY (50 µl sample size). Any result greater than 1,000 pg/ml in a 50 µl sample should be diluted using matrix solution and the assay repeated until the results fall within range.

XIII. GRAPH OF TYPICAL REFERENCE CURVE



Typical Standard Curve, not to be used to calculate data.

XIV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of NPY that can be detected by this assay is 3 pg/ml using a 50 µl sample size, as derived from Statistical Ligand Immunoassay Analysis of multiple assays (n = 6) calculating the mean plus 2 standard deviations of the minimal detectable concentrations.

XIV. ASSAY CHARACTERISTICS (continued)

B. Specificity

Human, Rat NPY	100%
Human, Rat NPY 2-36	67%
Human, Rat NPY 3-36	68%
Human, Rat NPY (Free Acid)	6%
Human, Rat NPY 1-24	0%
Human, Rat NPY 13-36	8%
Human, Rat NPY 18-36	0%
NPY 22-36	0%
(Leu^{31}, Pro^{34}) Human, Rat NPY	41%
Porcine NPY	44%
Porcine NPY 3-36	41%
Human PYY	0%
Human PYY 3-36	0%
Human PP	0%
Human GIP	0%
Human Ghrelin	0%
Des-Octanoyl Human Ghrelin	0%
Intact Human Proinsulin	0%
Glucagon	0%

C. Precision

Intra and Inter-Assay Variations

Sample ID	NPY (pg/ml) Mean, n = 5	Intra-assay CV (%)	Inter-assay CV (%)
Serum #18	19.6	3.89	8.12
Serum # 3	25.4	2.06	6.58
Serum #13	41.2	2.60	2.92
Serum # 2	47.1	4.27	4.46
Plasma # 6	16.8	3.84	9.40
Plasma # 12	19.9	3.28	8.82
Plasma # 15	26.7	3.66	13.5
Plasma # 14	35.8	3.13	8.62

Human serum and plasma samples in small aliquots frozen at -70°C are assayed for NPY. Intra-assay variations were calculated from results of five duplicate determinations in one assay. Inter-assay variations were calculated from results of five separate assays with duplicate samples in each assay.

XIV. ASSAY CHARACTERISTICS (continued)

D. Spike Recovery Rate of Human NPY in Assay Samples

Sample	I.D.	Basal NPY	Basal + 40 pg/ml Spiked NPY		Basal + 500 pg/ml Spiked NPY	
		pg/ml	pg/ml	Recovery	pg/ml	Recovery
Serum	# 19	12.8	51.4	96.5 %	501.4	97.6 %
	# 20	16.4	51.4	87.5 %	417.0	80.1 %
	# 25	27.7	73.3	114.0%	556.5	105.8 %
	# 26	141.6	180.6	97.5 %	636.6	99.0 %
% Recovery Rate Mean ± S.D.	----	100 %	98.9 % ± 11.1 %		95.6 % ± 11.0 %	
Plasma	# 19	5.9	45.1	98.0 %	503.4	99.5 %
	# 2	26.4	65.1	96.8 %	553.2	105.4 %
	# 15	10.1	45.5	88.5 %	479.7	93.9 %
	# 23	26.8	63.8	92.5 %	519.9	98.6 %
% Recovery Rate Mean ± S.D.	----	100 %	94.0 % ± 4.3 %		95.6 % ± 11.0 %	

Varying amounts of human NPY were added to individual human serum and plasma samples and the resulting NPY content of each sample was assayed by Human NPY ELISA. The recovery rate = [(Observed NPY concentration after spike – Basal NPY level) / spiked NPY concentration] x 100%.

XIV. ASSAY CHARACTERISTICS (continued)

E. Linearity of Sample Dilution

Sample	I. D.	Sample Volume						
		50 µl	30 µl		20 µl		10 µl	
		pg/ml	pg/ml	Expected	pg/ml	Expected	pg/ml	Expected
Serum	# 19	651	389	100%	264	102%	132	101%
	# 20	292	179	103%	120	103%	64.4	110%
	# 25	249	146	98%	98.8	99 %	48.8	98%
	# 26	153	91.2	99%	60.1	98 %	24.8	81%
% Expected Mean ± S.D.	----	100 %	99.8 % ± 2.0 %		100.4 % ± 2.3 %		97.7 % ± 12.3 %	
Plasma	# 19	565	350	103%	229	101%	116	102%
	# 2	352	204	96 %	131	93%	64.4	92%
	# 15	198	120	101%	80.8	102%	40.8	103%
	# 23	131	69.3	88%	44.8	85 %	22.1	84%
% Expected Mean ± S.D.	----	100 %	97.1 % ± 6.7 %		95.3 % ± 7.9 %		95.3 % ± 9.8 %	

Samples are spiked with human NPY to various levels and assayed by ELISA at the indicted volumes supplemented with Matrix Solution to a total of 50 µl per well. Measured NPY levels are corrected for various dilution factors and then divided by levels found at 50 µl sample size to obtain the % of expected values.

XV. NORMAL RANGE OF NPY LEVELS IN HUMAN BLOOD

The normal range of serum/plasma NPY in healthy human subjects has been reported to be within the range 5 ~ 90 pg/ml in the literatures

XVI. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com\bmia.

XVII. TROUBLESHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure all reagents and samples are added to the bottom of each well.
6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with HRP Wash Buffer or 3) overexposure to light after substrate has been added.

XVIII. REPLACEMENT REAGENTS

Reagents	**Cat. #**
Microtiter Plates	EPDAR
10X HRP Wash Buffer Concentrate (50 ml)	EWB-HRP
Human NPY Standard	E8025-K
Human NPY Quality Controls 1 and 2	E6025-K
Matrix Solution	EMTXD4
Assay Buffer	AB-PHK
Human NPY Capture Antibody	E1025-C
Human NPY Detection Antibody	E1025-D
Enzyme Solution	EHRP
Substrate	ESS-TMB2
Stop Solution	ET-TMB

XIX. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:

TOLL FREE US: (800) MILLIPORE

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

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C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.