Human AGF/ANGPTL6
96-Well Plate Assay
Cat. # EZHAGF-28K

HUMAN AGF/ANGPTL6 ELISA KIT
96-Well Plate (Cat. # EZHAGF-28K)

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HUMAN AGF/ANGPTL6 ELISA Kit
96-Well Plate (Cat.# EZHAGF-28K)

I. INTENDED USE

This kit is used for the non-radioactive quantification of AGF/ANGPTL6 in human serum and plasma. (This assay can be used to measure AGF in serum, plasma and tissue culture media samples. The levels of AGF in serum and plasma may not correlate well possibly due to AGF also being expressed in platelets**.**) One kit is sufficient to measure 38 unknown samples in duplicate. ***This kit is for Research Use Only. Not for Use in Diagnostic Procedures.***

II. PRINCIPLES OF ASSAY

This assay is a Sandwich ELISA based, sequentially, on: 1) capture of Human AGF molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of anti-Human AGF polyclonal antibody, 2) wash away of unbound materials from samples, 3) binding of a biotinylated anti-Human AGF polyclonal antibody to the captured molecules, 4) wash away of unbound materials from samples, 5) conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 6) wash away of free enzyme conjugates, and 7) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured Human AGF in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of Human AGF.

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 Human AGF 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 °C.

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 AGF/ANGPTL6 Standard

Human AGF/ANGPTL6 standard, lyophilized

Quantity: 0.5ml upon hydration

Preparation: Reconstitute with 0.5ml distilled or de-ionized water. See insert for concentration

5. Human AGF/ANGPTL6 Quality Controls 1 and 2

One vial each, lyophilized, containing human AGF/ANGPTL6 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: 2.5 ml/vial/ lyophilized

Preparation: Reconstitute vial with 2.5 ml de-ionized water before use.

III. REAGENTS SUPPLIED (CONTINUED)

7. Assay Buffer

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

Quantity: 12 ml/vial

Preparation: Ready to use.

8. Human AGF/ANGPTL6 Detection Antibody

Pre-titered detection antibody solution in buffer

Quantity: 12 ml/vial

Preparation: Ready to use.

9. Enzyme Solution

Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.

Quantity: 12 ml/vial

Preparation: Ready to use.

10. Substrate

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

Quantity: 12 ml/vial

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

11. Stop Solution

0.3 M HCl

Quantity: 12 ml/vial

Preparation: Ready to use.

[Caution: Corrosive Solution]

IV. STORAGE AND STABILITY

Recommended storage for kit components is 2-8°C.

All components are shipped and stored at 2-8°C. Reconstituted standards and controls can be frozen for future use but repeated freeze thaws should be avoided. 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 or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

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

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VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. 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^{o}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^{o}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 , 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.

   Customers need to determine the optimal dilution for their samples. Generally, samples require a 1:5 dilution in Matrix Solution provided in the kit (i.e. 40 ul matrix plus 10 ul sample).

VIII. REAGENT PREPARATION

A. Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human AGF/ANGPTL6 Standard with 0.5 ml distilled or de-ionized water to give a concentration described on the analysis sheet. Invert and mix gently until completely in solution.

2. Label six tubes as 1, 2, 3, 4, 5, and 6. Add 100 µL Assay Buffer to each of the six tubes. Perform 2 times serial dilutions by adding 100 µL of the reconstituted standard to Tube 6, mix well and transfer 100 µL from Tube 6 to Tube 5, mix well and transfer 100 µL from Tube 5 to Tube 4, mix well and transfer 100 µL from Tube 4 to Tube 3, mix well and transfer 100 µL from Tube 3 to Tube 2, mix well and transfer 100 µL from Tube 2 to Tube 1. Mix well.

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

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Stock Concentration (ng/mL)
0.5 mL	0	**X** (refer to analysis sheet for exact concentration)

Tube #	Volume of Assay Buffer to Add	Volume of Standard to Add	Standard Concentration (ng/mL)
Tube 6	0.1 mL	0.1 mL of reconstituted standard	**X/2**
Tube 5	0.1 mL	0.1 mL of Tube 6	**X/4**
Tube 4	0.1 mL	0.1 mL of Tube 5	**X/8**
Tube 3	0.1 mL	0.1 mL of Tube 4	**X/16**
Tube 2	0.1 mL	0.1 mL of Tube 3	**X/32**
Tube 1	0.1 mL	0.1 mL of Tube 2	**X/64**

B. Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each Human AGF/ANGPTL6 Quality Control 1 and Quality Control 2 with 0.50 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.

VIII. REAGENT PREPARATION (continued)

C. Matrix preperation

Use care in opening the lyophilized matrix vial. Reconstitute vial with 2.5 ml distilled or de-ionized water and gently invert to ensure complete hydration. Unused portions of the reconstituted matrix solution should be stored at ≤ -20°C. Avoid further freeze/thaw cycles

IX. HUMAN AGF/ANGPTL6 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 in duplicate 20 µl Matrix Solution to Blank, Standards and Quality Control wells (refer to § X. for suggested well orientations).

4. Add in duplicate 80 µl assay buffer to each of the Blank and sample wells.

5. Add in duplicate 60 µl assay buffer to each of the Standard and QC wells.

6. Add in duplicate 20 µl AGF/ANGPTL6 Standards in the order of ascending concentrations to the appropriate wells. Add in duplicate 20 µl QC1 and 20 µl QC2 to the appropriate wells. Add sequentially 20 µl of the diluted sample to the remaining wells.

7. Cover the plate with plate sealer and incubate at room temperature for 1.5 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.

8. 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.

IX. HUMAN AGF/ANGPTL6 ELISA ASSAY PROCEDURE (continued)

9. Add 100 µl Detection Antibody Solution to each well. Re-cover plate with sealer and incubate at room temperature for 1 hour on an orbital micro-titer plate shaker set to rotate at moderate speed, approximately 400-500 rpm.

10. 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.

11. 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.

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

13. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for **approximately** 5 to 20 minutes. Blue color should be formed in wells of the AGF/ANGPTL6 standards with intensity proportional to increasing concentrations of AGF/ANGPTL6.

    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.

14. 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 AGF/ANGPTL6 ELISA Kit (Cat. # EZHAGF-28K)

	Step 1	Step 2	Step 3	Step 4-5	Step 6	Step 7-8	Step 9	Step 10	Step 11	Step 12	Step 13-14			
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	Seal, Agitate, Incubate 1.5 hrs at Room Temperature. Wash 3X with 300 µL Wash Buffer.	Detection Antibody	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 3X with 300 µL Wash Buffer.	Substrate	Seal, Agitate, Incubate 5~20 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1			20 µL	80 µL	--		100 µL		100 µL		100 µL		100 µL	
C1, D1			20 µL	60 µL	20 µL of Tube 1									
E1, F1			20 µL	60 µL	20 µL of Tube 2									
G1, H1			20 µL	60 µL	20 µL of Tube 3									
A2, B2			20 µL	60 µL	20 µL of Tube 4									
C2, D2			20 µL	60 µL	20 µL of Tube 5									
E2, F2			20 µL	60 µL	20 µL of Tube 6									
G2, H2			20 µL	60 µL	20 µL of reconstituted Standard									
A3, B3			20 µL	60 µL	20 µL of QC 1									
C3, D3			20 µL	60 µL	20 µL of QC 2									
E3, F3			--	80 µL	20 µL of Sample									
G3, H3 Etc.			--	80 µl	20 µl of Sample									

X. MICROTITER PLATE ARRANGEMENT

Human AGF/ANGPTL6 ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 4	QC1	Etc.								
B	Blank	Tube 4	QC1	Etc.								
C	Tube 1	Tube 5	QC2									
D	Tube 1	Tube 5	QC2									
E	Tube 2	Tube 6	Sample 1									
F	Tube 2	Tube 6	Sample 1									
G	Tube 3	Reconstituted Standard	Sample 2									
H	Tube 3	Reconstituted Standard	Sample 2									

XI. CALCULATIONS

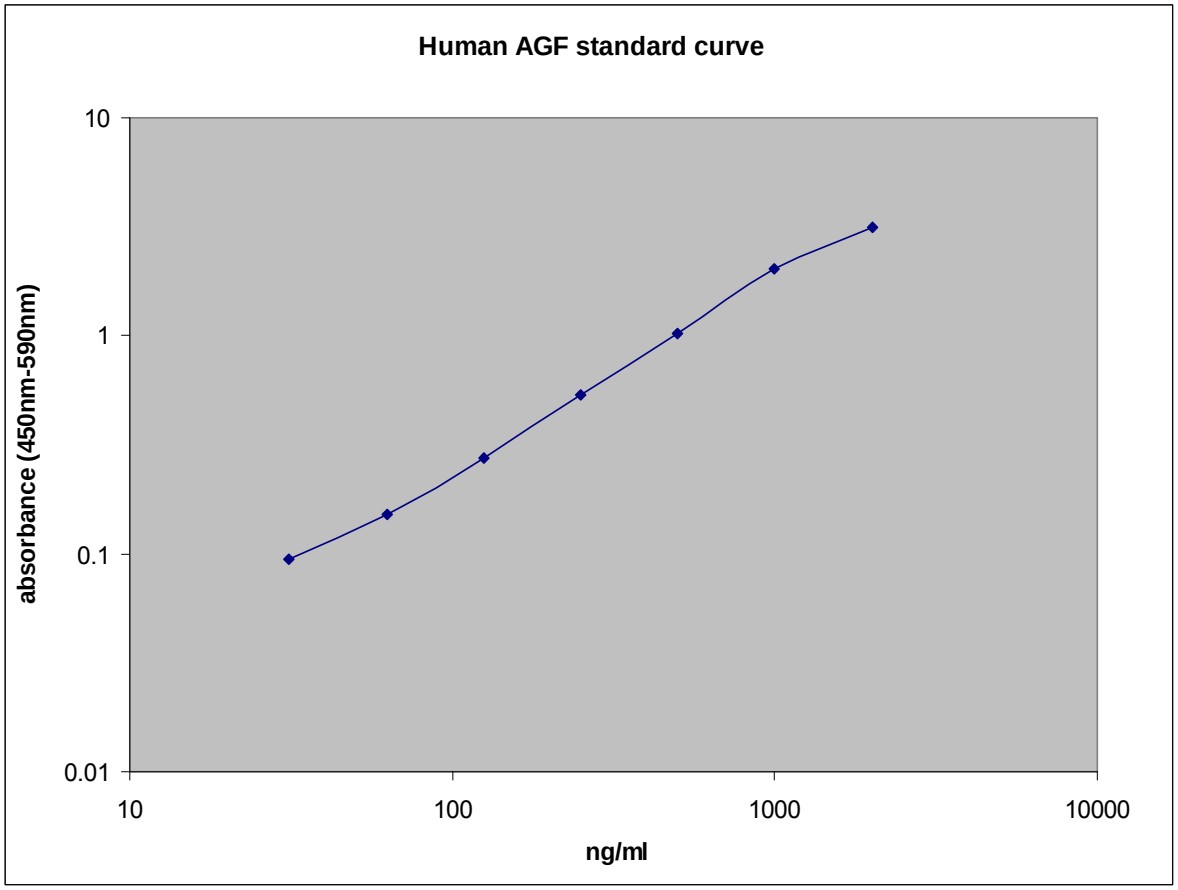
The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function

Note: When sample volumes assayed differ from 20 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 µl of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 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 5.0 ng/ml AGF/ANGPTL6 (20 µl sample size).
4. The approximate range of this assay is 31.25 ng/ml to 2,000 ng/ml AGF/ANGPTL6 (20 µl sample size). Any result greater than 2,000 ng/ml in a 20 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 AGF/ANGPTL6 that can be detected by this assay is 5.0 ng/ml using a 20 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

The antibody pair used in this assay is specific to Human AGF and has no significant cross-reactivity with other Human ANGPTLS or Liver Proteins.

C. Precision

Intra-Assay Variation

Sample No.	Mean AGF Levels (ng/mL)	Intra-Assay %CV
1	132.5	1.21%
2	482.1	1.81%

Inter-Assay Variation

Sample No.	Mean AGF Levels (ng/mL)	Inter-Assay %CV
1	135.2	8.52%
2	514	11.02%

The assay variations of Millipore Human AGF ELISA kits were studied on two samples at two levels on AGF standard curve. The mean intra-assay variation was calculated from results of eight determinations of the indicated samples. The mean inter-assay variations of each sample was calculated from results of six separate assays with duplicate samples in each assay.

XIV. ASSAY CHARACTERISTICS (continued)

D. Spike Recovery Rate of Human AGF/ANGPTL6 in Assay Samples

sample No.	AGF Added (ng/mL)	Expected (ng/mL)	Observed (ng/mL)	% of Recovery
1	0	49		
	125	174	174	100 %
	250	299	304	102%
	500	549	545	99%
2	0	113		
	125	238	237	99 %
	250	363	366	101%
	500	613	609	99%
3	0	126		
	125	251	256	104%
	250	376	384	103%
	500	626	622	99%
4	0	134		
	125	259	254	96%
	250	384	378	98%
	500	634	611	95%
5	0	83		
	125	208	203	96%
	250	333	325	97%
	500	583	567	97%
Average				99 %

Varying amounts of human AGF/ANGPTL6 were added to individual human serum and plasma samples and the resulting AGF/ANGPTL6 content of each sample was assayed by Human AGF/ANGPTL6 ELISA. The recovery rate = [(Observed AGF/ANGPTL6 concentration after spike – Basal AGF/ANGPTL6 level) / spiked AGF/ANGPTL6 concentration] x 100%.

XIV. ASSAY CHARACTERISTICS (continued)

E. Linearity of Sample Dilution

Effect of Serum Dilution

Sample No.	Volume Sampled (µL)	Expected (ng/mL)	Observed (ng/mL)	% Of Expected
1	20	220		
	10	110	115	95%
	5	55	57	91%
	2.5	27.5	32	87%
2	20	211		
	10	105.5	116	88%
	5	52.75	58	80%
3	20	212		
	10	106	104	94%
	5	53	56	93%
	2.5	26.5	30	98%
4	20	200		
	10	100	106	95%
	5	50	53	81%
5	20	209		
	10	104.5	108	111%
	5	52.25	55	95%
	2.5	26.125	29	94%
Average				107%

Five human plasma samples with the indicated sample volumes were assayed. Required amounts of serum matrix were added to compensate for lost volumes below 20 µL. The resulting dilution factors of neat, 2, 4, and 8 representing 20 µL, 10 µL, 5 µL, and 2.5 µL sample volumes assayed, respectively, were applied in the calculation of observed AGF concentrations. % expected = observed/expected x 100%.

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/techlibrary/index.do.

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	EP28
10X HRP Wash Buffer Concentrate (50 ml)	EWB-HRP
Human AGF/ANGPTL6 Standard	E8028-K
Human AGF/ANGPTL6 Quality Controls 1 and 2	E6028-K
Matrix Solution	EMTX-RS2
Assay Buffer	EABGLP
Human AGF/ANGPTL6 Detection Antibody	E1028
Enzyme Solution	EHRP-5
Substrate	ESS-TMB3
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: EMD Millipore Corporation
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.

B. Conditions of Sale

For Research Use Only. Not for Use in Diagnostic Procedures.

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.