Quantikine® ELISA

Human CXCL5/ENA-78 Immunoassay

Catalog Number DX000

For the quantitative determination of human Epithelial Neutrophil Activating peptide 78 (ENA-78) concentrations in cell culture supernate, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Epithelial Neutrophil Activating peptide 78 (ENA-78) is a CXC chemokine (CXCL5) that was originally isolated from media conditioned by the growth of a human lung type-II alveolar epithelial cell line (A549) stimulated by IL-1β or TNF-α (1). The full-length cDNA encodes a 114 amino acid (aa) residue precursor protein with a 36 aa residue signal peptide that is cleaved to generate the 78 aa residue secreted protein. ENA-78 shares significant amino acid sequence identity with NAP-2 (53%), GROα, β, and γ (52%, 48% and 51%, respectively), and IL-8 (22%). The gene for ENA-78 has been mapped to chromosome 4q13-q21 (2, 3).

Like other CXC chemokines, ENA-78 is a neutrophil attractant and activator *in vitro* (4). Based on cross-desensitization experiments, it has been suggested that ENA-78 activity can be mediated through the IL-8 receptor system (1). ENA-78 is expressed in human platelets (5). The expression of ENA-78 has also been detected in neutrophils and monocytes/macrophages following LPS stimulation. In addition, ENA-78 expression is highly inducible in endothelial cells, vascular smooth muscle cells, epithelial cells and pulmonary fibroblasts by pro-inflammatory cytokines such as IL-1β or TNF-α (6, 7). Increased ENA-78 expression has also been found to be associated with neutrophil influx in various inflammatory conditions (7-9).

The Quantikine Human CXCL5/ENA-78 Immunoassay is a 4.5 hour solid phase ELISA designed to measure human ENA-78 in cell culture supernate, serum, and plasma. It contains *E. coli*-expressed recombinant human ENA-78 and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant human ENA-78 accurately. Results obtained using natural human ENA-78 showed dose curves that were parallel to the standard curves obtained using the recombinant Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human ENA-78.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human ENA-78 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any ENA-78 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human ENA-78 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of ENA-78 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, further dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human ENA-78 Microplate	890480	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human ENA-78.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human ENA-78 Standard	890482	20 ng of recombinant human ENA-78 in a buffered protein base with preservative; lyophilized.	Aliquot and store for up to 1 month at ≤ -20 °C.* Avoid repeated freeze-thaw cycles.
Human ENA-78 Conjugate	890481	21 mL of polyclonal antibody specific for human ENA-78 conjugated to horseradish peroxidase with preservative.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	2 vials (11 mL/vial) of a buffered protein base with preservative.	
Calibrator Diluent RD5L	895028	21 mL of a concentrated buffered protein base with preservative. *For cell culture supernate samples. Used diluted 1:5 in this assay.*	
Calibrator Diluent RD6-1	895163	21 mL of animal serum with preservative. *For serum/plasma samples. Used undiluted in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.
- Human ENA-78 Controls (optional; available from R&D Systems).

PRECAUTIONS

Calibrator Diluent RD6-1 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

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SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Normal human serum added to cell culture media may contain high levels of ENA-78. For best results, do not use normal human serum for growth of cell cultures if assaying for ENA-78 production. Because there is no species cross-reactivity of this kit, human ENA-78 levels in culture media containing 10% bovine or fetal bovine serum can be assayed without interference.*

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Platelet-Poor Plasma - Collect plasma on ice using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the separated plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

ENA-78 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of ENA-78, platelet-poor plasma should be used. It should be noted that many protocols for plasma preparation, including procedures recommended by the National Committee for Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.

SAMPLE PREPARATION

Cell culture supernate samples require at least a 10-fold dilution prior to assay. A suggested 10-fold dilution is 15 μL of sample + 135 μL of Calibrator Diluent RD5L (diluted 1:5).*

Serum samples require at least at 2-fold dilution prior to assay. A suggested 2-fold dilution is 70 μL of sample + 70 μL of Calibrator Diluent RD6-1.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

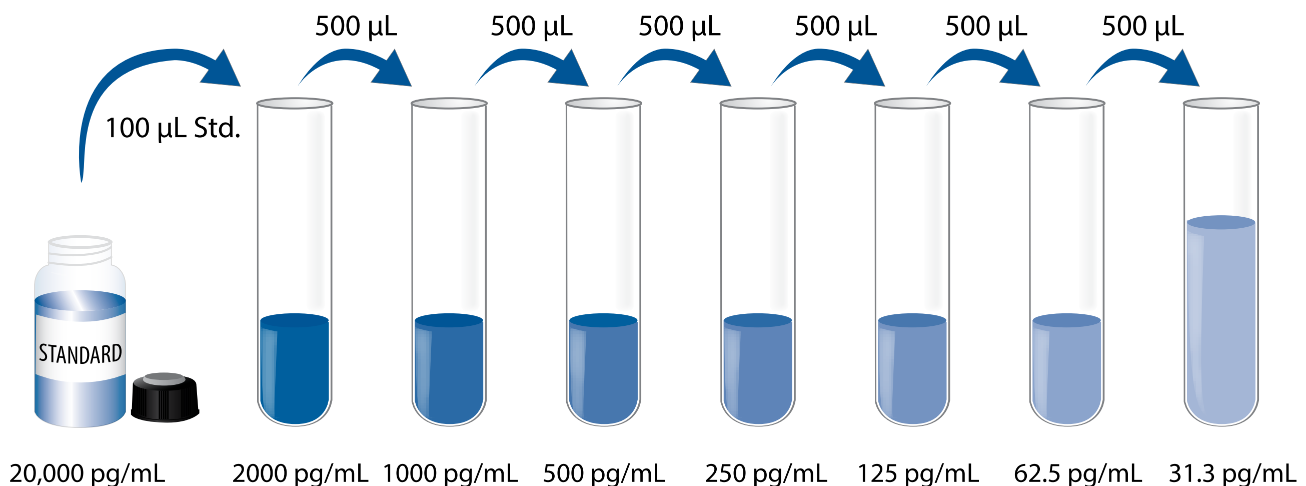
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

Calibrator Diluent RD5L (diluted 1:5) - **For cell culture supernate samples only.** Add 20 mL of Calibrator Diluent RD5L to 80 mL deionized or distilled water to yield 100 mL of Calibrator Diluent RD5L (diluted 1:5).

Human ENA-78 Standard - Reconstitute the Human ENA-78 Standard with 1 mL of deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 μL of Calibrator Diluent RD5L (diluted 1:5) (*for cell culture supernate samples*) or Calibrator Diluent RD6-1 (*for serum/plasma samples*) into the 2000 pg/mL tube. Pipette 500 μL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



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ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 200 µL of Assay Diluent RD1W to each well.
4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper toweling.
6. Add 200 µL of Human ENA-78 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

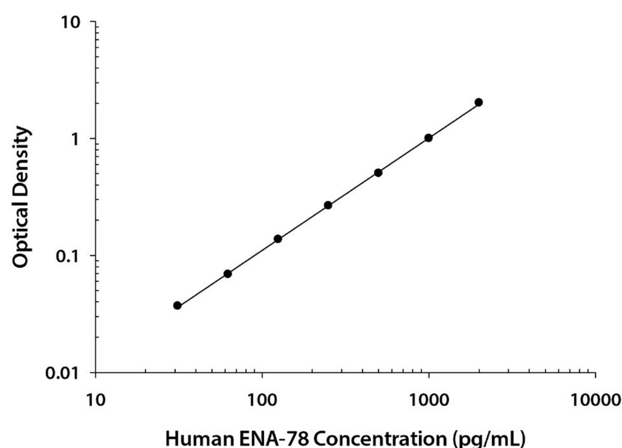
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human ENA-78 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

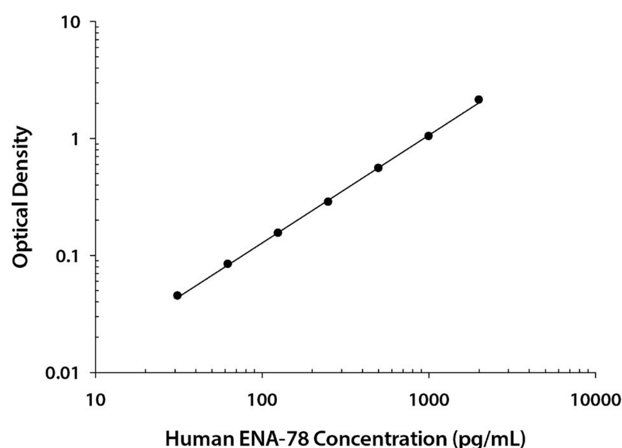
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.039 0.039	0.039	—
31.3	0.077 0.076	0.076	0.037
62.5	0.109 0.107	0.108	0.069
125	0.175 0.177	0.176	0.137
250	0.308 0.303	0.306	0.267
500	0.544 0.542	0.543	0.504
1000	1.068 1.012	1.040	1.001
2000	2.080 2.031	2.056	2.017

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.036 0.036	0.036	—
31.3	0.083 0.079	0.081	0.045
62.5	0.123 0.117	0.120	0.084
125	0.194 0.188	0.191	0.155
250	0.321 0.321	0.321	0.285
500	0.604 0.575	0.590	0.554
1000	1.102 1.051	1.076	1.040
2000	2.236 2.095	2.166	2.130

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PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	112	251	1096	129	278	1085
Standard deviation	6.0	14.6	44.9	12.6	26.8	96.5
CV (%)	5.4	5.8	4.1	9.8	9.6	8.9

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	113	244	995	109	240	989
Standard deviation	9.4	12.9	37.8	10.1	17.8	65.8
CV (%)	8.3	5.3	3.8	9.3	7.4	6.7

RECOVERY

The recovery of human ENA-78 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=5)	101	95-107%
Serum (n=5)	101	93-109%
EDTA plasma (n=5)	98	92-104%
Heparin plasma (n=5)	98	93-103%
Citrate plasma (n=5)	100	94-106%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human ENA-78 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=5)	Serum (n=5)	EDTA plasma (n=5)	Heparin plasma (n=5)	Citrate plasma (n=5)
1:2	Average % of Expected	94	99	98	100	100
	Range (%)	91-97	96-103	90-107	92-104	94-104
1:4	Average % of Expected	91	101	99	101	99
	Range (%)	88-94	97-107	92-106	90-106	94-103
1:8	Average % of Expected	94	94	99	100	100
	Range (%)	89-98	90-98	90-109	91-109	96-108
1:16	Average % of Expected	101	93	100	101	104
	Range (%)	92-109	86-98	90-113	91-114	95-108

SENSITIVITY

The minimum detectable dose (MDD) of human ENA-78 is typically less than 15 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human ENA-78 produced at R&D Systems.

SAMPLE VALUES

Serum/Plateler-poor Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human ENA-78 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=39)	1449	553-2954	589

Sample Type	Mean of Detectable (pg/mL)	% Detetable	Range (pg/mL)
Platelet-poor EDTA plasma (n=40)	227	98	ND-902
Platelet-poor heparin plasma (n=40)	115	80	ND-879
Platelet-poor citrate plasma (n=40)	99	98	ND-292

ND=Non-detectable

Cell Culture Supernates - Human peripheral blood mononuclear cells (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of natural human ENA-78.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	5650	6000
Stimulated	13,210	23,620

SPECIFICITY

This assay recognizes natural and recombinant human ENA-78.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range rhENA-78 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:	Recombinant mouse:
CXCL8/IL-8	GM-CSF
GROα	IL-1α
GROβ	IL-1β
GROγ	IL-3
MCP-1	IL-4
MIP-1α	IL-5
MIP-1β	IL-6
PTN	IL-7
RANTES	IL-9
	IL-10
	IL-13
	LIF
	MIP-1α
	MIP-1β
	SCF
	TNF-α

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

NOTES

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