

# Quantikine<sup>®</sup>

## Human 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 that was originally isolated from media conditioned by the growth of a human lung type-II alveolar epithelial cell line (A549) stimulated by IL-1 $\beta$  or TNF- $\alpha$  (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%), GRO $\alpha$ ,  $\beta$ , and  $\gamma$  (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-1 $\beta$  or TNF- $\alpha$  (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 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 the Quantikine Human ENA-78 Immunoassay 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 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 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, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- As manufacturers we take great care to ensure that our products are suitable for use with all validated sample types, as designated in the product insert. However, it is possible that in some cases, high levels of interfering factors may cause unusual results.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Soluble receptors or other binding proteins present in biological samples do not necessarily interfere with the measurement of ligands in samples. Until the factors have been tested in the Quantikine Immunoassay, however, the possibility of interference cannot be excluded.

## MATERIALS PROVIDED

**ENA-78 Microplate** (Part 890480) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against ENA-78.

**ENA-78 Conjugate** (Part 890481) - 21 mL of polyclonal antibody against ENA-78 conjugated to horseradish peroxidase with preservative.

**ENA-78 Standard** (Part 890482) - 20 ng of recombinant human ENA-78 in a buffered protein base with preservative; lyophilized.

**Assay Diluent RD1W** (Part 895117) - 2 vials (11 mL/vial) of a buffered protein base with preservative.

**Calibrator Diluent RD5L Concentrate (5X)** (Part 895028) - 21 mL of a buffered protein base with preservative. *For cell culture supernate samples.*

**Calibrator Diluent RD6-1** (Part 895163) - 21 mL of animal serum with preservative. *For serum/plasma samples.*

**Wash Buffer Concentrate** (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.

**Color Reagent A** (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

**Color Reagent B** (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

**Stop Solution** (Part 895032) - 6 mL of 2 N sulfuric acid.

**Plate Covers** - 4 Adhesive strips.

## STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Calibrator Diluent RD5L (1X)	
	Calibrator Diluent RD6-1	
	Assay Diluent RD1W	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	Aliquot and store for up to 1 month at ≤ -20° C.* Avoid repeated freeze-thaw cycles.
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*

\*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.
- 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. Wear eye, hand, face, and clothing protection when using this material.

## SAMPLE COLLECTION AND STORAGE

**Cell Culture Supernates** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles. **Caution:** *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 one hour at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Plasma** - Collect plasma on ice using citrate, EDTA, or heparin 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  $\leq -20^{\circ}$  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-free plasma should be used. It should be noted that many protocols for plasma preparation, including procedures recommended by the National Committee for Clinical Laboratory Standards (NCCLS), 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

All serum samples require at least a 2-fold dilution into Calibrator Diluent RD6-1. A suggested 2-fold dilution is 70  $\mu\text{L}$  sample + 70  $\mu\text{L}$  Calibrator Diluent RD6-1.

All cell culture supernate samples require at least a 10-fold dilution into Calibrator Diluent RD5L (1X). A suggested 10-fold dilution is 15  $\mu\text{L}$  sample + 135  $\mu\text{L}$  Calibrator Diluent RD5L (1X).

## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

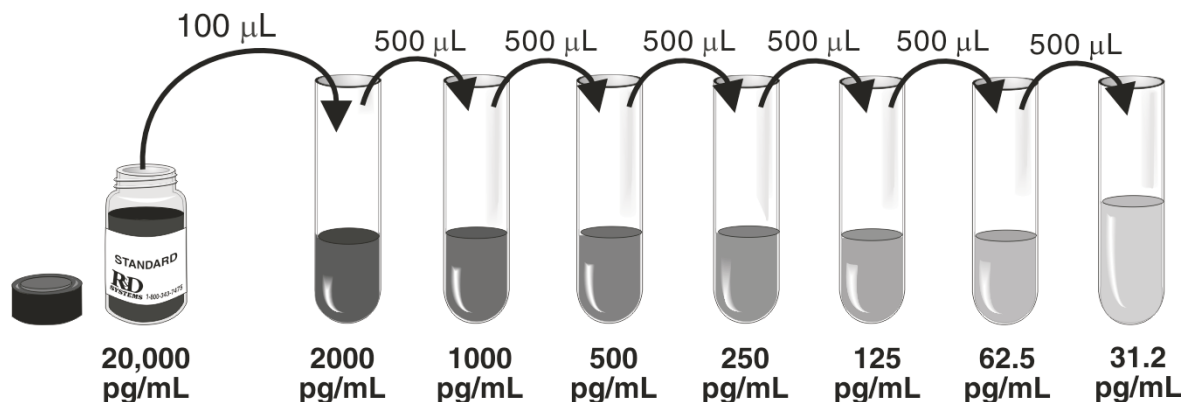
**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200  $\mu\text{L}$  of the resultant mixture is required per well.

**Calibrator Diluent RD5L (1X)** - Dilute 20 mL of Calibrator Diluent RD5L Concentrate (5X) into deionized or distilled water to yield 100 mL of Calibrator Diluent RD5L (1X).

**ENA-78 Standard** - Reconstitute the 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  $\mu\text{L}$  of the appropriate Calibrator Diluent (*Calibrator Diluent RD5L (1X) for cell culture supernate samples or Calibrator Diluent RD6-1 for serum/plasma samples*) into the 2000 pg/mL tube. Pipette 500  $\mu\text{L}$  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).



## 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  $\mu\text{L}$  of Assay Diluent RD1W to each well.
4. Add 50  $\mu\text{L}$  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  $\mu\text{L}$ ) 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  $\mu\text{L}$  of 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  $\mu\text{L}$  of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50  $\mu\text{L}$  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 as directed in the Sample Preparation section.

## ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, standards and samples as instructed.



2. Add 200  $\mu\text{L}$  Assay Diluent RD1W to each well.



3. Add 50  $\mu\text{L}$  Standard, control, or sample\* to each well.  
Incubate 2 hours at RT.



4. Aspirate and wash 3 times.



5. Add 200  $\mu\text{L}$  Conjugate to each well.  
Incubate 2 hours at RT.



6. Aspirate and wash 3 times.



7. Add 200  $\mu\text{L}$  Substrate Solution to each well.  
Incubate 30 minutes at RT. **Protect from light.**



8. Add 50  $\mu\text{L}$  Stop Solution to each well.  
Read at 450 nm within 30 minutes.  
 $\lambda$  correction 540 or 570 nm

\*Serum and cell culture supernate samples require dilution.

# CALCULATION OF RESULTS

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

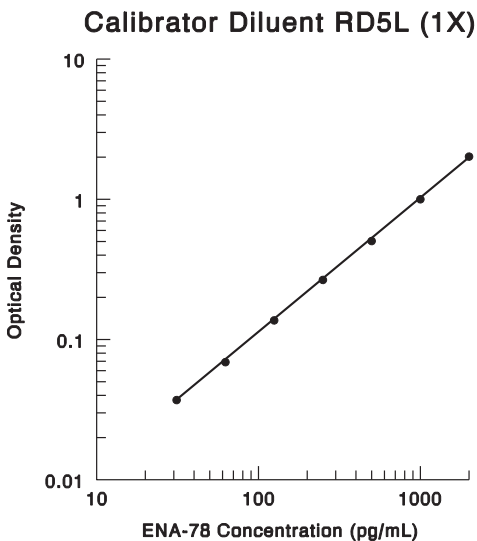
Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

To determine the ENA-78 concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding ENA-78 concentration.

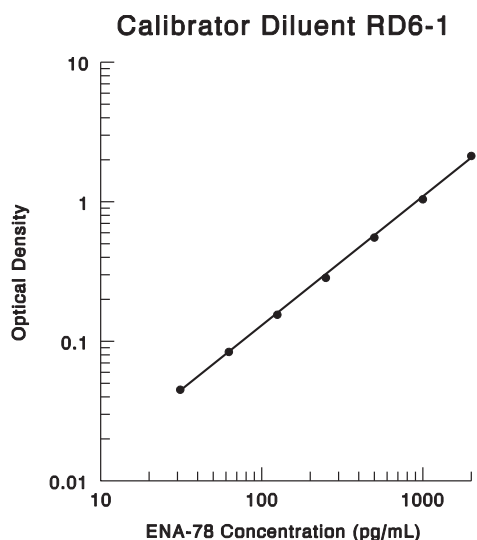
If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

## TYPICAL DATA

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



(pg/mL)	O.D.	Average	Corrected
0	0.039 0.039	0.039	—
31.2	0.077 0.109	0.076	0.037
62.5	0.107 0.175	0.108	0.069
125	0.177 0.308	0.176	0.137
250	0.303 0.544	0.306	0.267
500	0.542 1.068	0.543	0.504
1000	1.012 2.080	1.040	1.001
2000	2.031	2.056	2.017



(pg/mL)	O.D.	Average	Corrected
0	0.036 0.036	0.036	—
31.2	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

## 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.
- 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.

## PRECISION

### Intra-assay Precision (Precision within an assay)

Three samples of known concentration were assayed twenty times on one plate to assess intra-assay precision.

### Inter-assay Precision (Precision between assays)

Three samples of known concentration were assayed in forty separate assays to assess inter-assay precision.

### Serum/Plasma Assay

Sample	Intra-assay Precision			Inter-assay Precision		
	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

### Cell Culture Supernate Assay

Sample	Intra-assay Precision			Inter-assay Precision		
	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

## RECOVERY

The recovery of ENA-78 spiked to three different levels in five samples throughout the range of the assay in various matrices was evaluated.

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

## CALIBRATION

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

## LINEARITY

To assess the linearity of the assay, five samples containing or spiked with high concentrations of ENA-78 in various matrices were diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media	Serum	EDTA plasma	Heparin plasma	Citrate plasma
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 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.

## SAMPLE VALUES

**Serum/Plasma** - Samples from apparently healthy volunteers were evaluated for the presence of 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)
Serum (n=40)	1449	589 - 2627
<b>platelet-poor plasma</b>		
EDTA Plasma (n=12)	161	29 - 427
Citrate Plasma (n=12)	45	29 - 71
Heparin Plasma (n=12)	342	92 - 644

It is important to note that sample collection and handling procedures will have a significant impact on measured ENA-78 levels. Refer to Sample Collection and Storage section for the recommended sample collection procedures.

**Cell Culture Supernates** - Human peripheral blood mononuclear cells ( $5 \times 10^6$  cells/mL) were cultured in RPMI supplemented with 5% fetal calf serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10  $\mu$ g/mL PHA for 1 and 5 days.

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

## SPECIFICITY

This assay recognizes recombinant and natural human ENA-78. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5L (1X) and Calibrator Diluent RD6-1, 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:

ANG	IL-1 sRII
AR	IL-2
CNTF	IL-2 sR $\alpha$
$\beta$ -ECGF	IL-2 sR $\beta$
EGF	IL-2 sR $\gamma$
Epo	IL-3
FGF acidic	IL-3 sR $\alpha$
FGF basic	IL-4
FGF-4	IL-4 sR
FGF-5	IL-5
FGF-6	IL-5 sR $\alpha$
G-CSF	IL-5 sR $\beta$
G-CSF R	IL-6
GM-CSF	IL-6 sR
GRO $\alpha$	IL-7
GRO $\beta$	IL-8
GRO $\gamma$	IL-9
HB-EGF	IL-10
HGF	IL-11
IFN- $\gamma$	IL-12
IGF-i	IL-13
IGF-II	KGF (FGF-7)
IL-1 $\alpha$	LAP (TGF- $\beta$ 1)
IL-1 $\beta$	LIF
IL-1ra	LIF R
IL-1 sRI	MCP-1

M-CSF
MIP-1 $\alpha$
MIP-1 $\beta$
$\beta$ -NGF
OSM
PD-ECGF
PDGF-AA
PDGF-AB
PDGF-BB
PTN
RANTES
SCF
SLPI
TGF- $\alpha$
TGF- $\beta$ 1
TGF- $\beta$ 3
TGF- $\beta$ 5
TGF- $\beta$ sRII
TNF- $\alpha$
TNF- $\beta$
sTNF RI
sTNF RII
VEGF

### Recombinant mouse:

GM-CSF
IL-1 $\alpha$
IL-1 $\beta$
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-13
LIF
MIP-1 $\alpha$
MIP-1 $\beta$
SCF
TNF- $\alpha$

### Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF- $\beta$ 1
porcine TGF- $\beta$ 1
porcine TGF- $\beta$ 2

## REFERENCES

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# PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H