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R&D SYSTEMS

Saliva Sensitive

Quantikine® ELISA

Human TRAIL/TNFSF10 Immunoassay

Catalog Number DTRL00

For the quantitative determination of human TNF-Related Apoptosis-Inducing Ligand (TRAIL) concentrations in cell culture supernates, cell lysates, serum, plasma, and saliva.

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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Manufactured and Distributed by:

USA R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413
TEL: 800 343 7475 612 379 2956
FAX: 612 656 4400
E-MAIL: info@bio-techne.com

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373 (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

INTRODUCTION

TNF-Related Apoptosis-Inducing Ligand (TRAIL), also called Apoptosis 2 Ligand (Apo2L) for its similarity in sequence, structure, and function to Fas Ligand/Apo1L, is a tumor necrosis factor superfamily (TNFSF) member designated TNFSF10 (1-3). TRAIL was cloned from human heart atrium, peripheral blood lymphocyte, and placenta cDNA libraries based on its similarity to regions highly conserved in the TNFSF (1). TRAIL is a 281 amino acid (aa), approximately 32 kDa, Type II transmembrane protein expressed on the cell surface (1, 2). It lacks a signal sequence, has a highly conserved and singly glycosylated C-terminal extracellular domain, a transmembrane domain, and a short N-terminal cytoplasmic domain. Like other members of the TNFSF, TRAIL also exists in a soluble form. SDS-PAGE analysis indicates that soluble TRAIL has an apparent molecular weight of approximately 24-28 kDa while gel filtration analysis suggests that it exists as a trimer of approximately 66-80 kDa (1, 2). Crystallography studies confirm that TRAIL is a homotrimeric jelly roll protein, but unlike other TNFSF members, TRAIL contains a zinc ion within the trimer interface (4). Three inward-facing Cys 230 residues coordinate the zinc ion. These residues and the zinc ion are crucial for TRAIL trimer stability, receptor binding, and function (4, 5). Within the TNFSF, TRAIL is most closely related to Fas Ligand with 28% aa identity. Across species, TRAIL is 65% identical at the aa level to its mouse homolog (1).

TRAIL has an extremely broad expression pattern based on Northern blot analysis. It is expressed in fetal kidney, liver, and lung, as well as in adult colon, heart, kidney, lung, ovary, peripheral blood lymphocytes, placenta, prostate, skeletal muscle, small intestine, spleen, and thymus (1, 2). TRAIL is variably expressed in tumor cell lines (1). Both cell-surface and soluble TRAIL induce apoptosis in a variety of lymphoid and non-lymphoid tumor cell lines (1, 2). These effects are mediated through binding TRAIL receptors 1 and 2 (TRAIL R1 [DR4] and TRAIL R2 [DR5]), both of which are expressed in many tissues as well. TRAIL also binds TRAIL R3 (DcR1), TRAIL R4 (DcR2), and osteoprotegrin (OPG), but is not able to affect apoptosis through these receptors. TRAIL R3, TRAIL R4, and OPG have become known as decoy receptors and are thought to be critical to the regulation of TRAIL signaling by competing for TRAIL binding. Differential sensitivities to TRAIL-induced apoptosis may be due to the balance of TRAIL receptor and TRAIL decoy receptor expression patterns on particular cells as well as the relative expression of intracellular signaling regulatory proteins (6, 7). Since TRAIL is capable of preferentially inducing apoptosis in tumor cells over normal cells, it has become an exciting prospect as a cancer chemotherapeutic (7).

The Quantikine® Human TRAIL/TNFSF10 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human TRAIL in cell culture supernates, cell lysates, serum, plasma, and saliva. It contains recombinant human TRAIL and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human TRAIL showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural human TRAIL.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TRAIL has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TRAIL present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human TRAIL 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 TRAIL 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.
- Any variation in 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 TRAIL Microplate	892373	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human TRAIL.	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 TRAIL Standard	892375	Recombinant human TRAIL in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer. Avoid repeated freeze-thaw cycles.*
Human TRAIL Conjugate	892374	21 mL of a polyclonal antibody specific for human TRAIL conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1S	895137	11 mL of a buffered protein base with preservatives.	
Cell Lysis Buffer 3	895366	21 mL of a buffered solution with preservatives. *Use diluted 1:5 in this assay. May turn yellow over time.*	
Calibrator Diluent RD5-33	895813	21 mL of a buffered protein base with preservatives. *Use undiluted for serum/plasma samples. Use diluted 1:4 for cell culture supernate/ cell lysate/saliva samples.*	
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.
- 50 mL, 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Centrifuge.
- Collection device for saliva samples which has no enzyme binding or filtering capabilities such as Salivette® or equivalent.
- Cold PBS for cell lysis.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human TRAIL Controls (optional; R&D Systems®, Catalog # QC177).

PRECAUTIONS

TRAIL is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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

Some components in this kit contain a preservative 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. Refer to the SDS on our website prior to use.

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.

Cell Lysates - Cells must be lysed prior to assay as directed in the Cell Lysis Procedure.

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.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Grossly hemolyzed samples are not suitable for use in this assay.

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

SAMPLE PREPARATION

Use polypropylene tubes.
Saliva samples require a 2-fold dilution. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5-33 (diluted 1:4)*.

CELL LYSIS PROCEDURE

Use polypropylene tubes.
Cultured cells must be lysed before assaying according to the following directions:

1. Wash cells three times in cold PBS.
2. Re-suspend cells in Cell Lysis Buffer 3 (diluted 1:5)* to a concentration of 1 x 10^7 cells/mL.
3. Incubate at 37 °C for 30 minutes with gentle mixing.
4. Centrifuge cells at 500 x g for 15 minutes.
5. Assay the supernate immediately or aliquot and store at ≤ -70 °C.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of TRAIL are found in saliva. We recommend using a face mask and gloves to protect kit reagents from contamination.*

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 480 mL of 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.

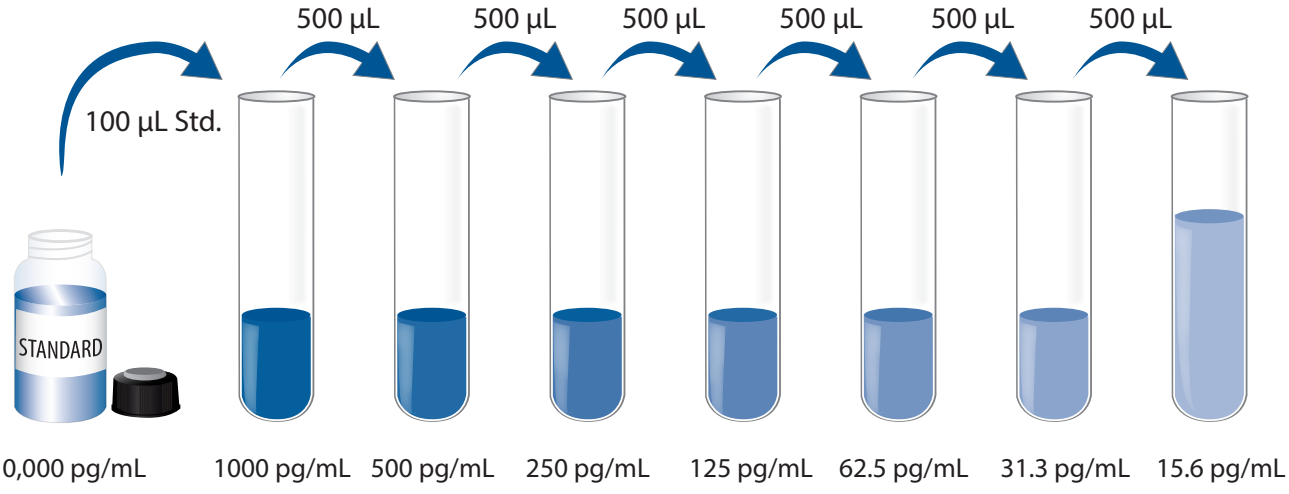
Cell Lysis Buffer 3 (diluted 1:5) - Add 20 mL of Cell Lysis Buffer 3 to 80 mL deionized or distilled water to prepare 100 mL of Cell Lysis Buffer 3 (diluted 1:5).

Calibrator Diluent RD5-33 (diluted 1:4) - **For cell culture supernate/cell lysate/saliva samples.** Add 5.0 mL of Calibrator Diluent RD5-33 to 15 mL of deionized or distilled water to prepare 20 mL of Calibrator Diluent RD5-33 (diluted 1:4).

Note: *Use undiluted Calibrator Diluent RD5-33 for serum and plasma samples.*

Human TRAIL Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human TRAIL Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD5-33 (*for serum/plasma samples*) or Calibrator Diluent RD5-33 (diluted 1:4) (*for cell culture supernate/cell lysate/saliva samples*) into the 1000 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 1000 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 standards, samples, and controls be assayed in duplicate.

Note: *High concentrations of TRAIL are found in saliva. We recommend using a face mask and gloves to protect kit reagents from contamination.*

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 100 µL of Assay Diluent RD1S to each well.
4. Add 50 µL of standard, sample* or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four 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 towels.
6. Add 200 µL of Human TRAIL Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
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 **on the benchtop. 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 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 may require dilution and/or lysis. See Sample Preparation or Cell Lysis Procedure.

CALCULATION OF RESULTS

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

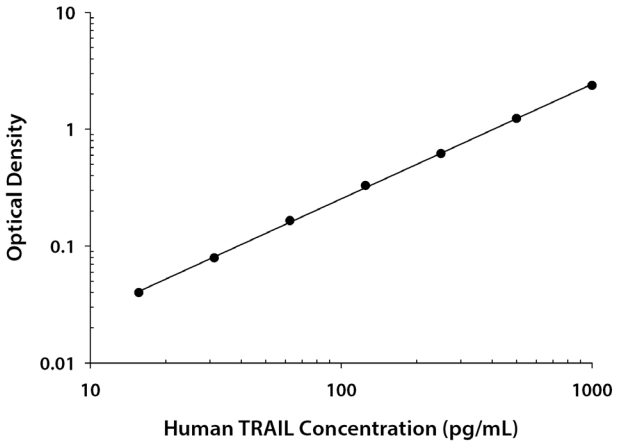
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points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

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

TYPICAL DATA

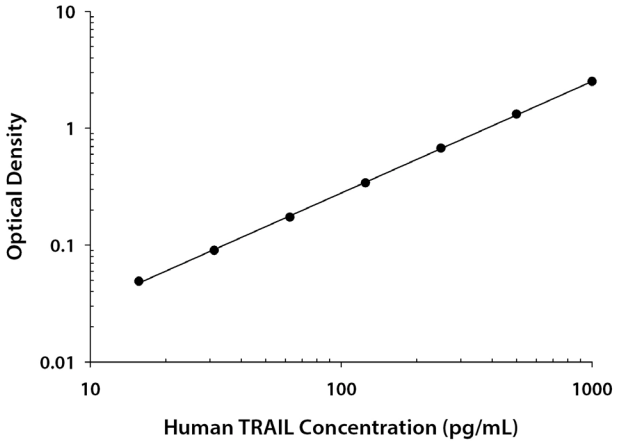
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/CELL LYSATE/SALIVA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.036 0.034	0.035	—
15.6	0.082 0.086	0.084	0.049
31.3	0.123 0.127	0.125	0.090
62.5	0.207 0.208	0.208	0.173
125	0.376 0.376	0.376	0.341
250	0.691 0.726	0.709	0.674
500	1.300 1.413	1.357	1.322
1000	2.515 2.572	2.544	2.509

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.039 0.034	0.037	—
15.6	0.079 0.073	0.076	0.039
31.3	0.114 0.117	0.116	0.079
62.5	0.200 0.202	0.201	0.164
125	0.358 0.375	0.367	0.330
250	0.629 0.682	0.656	0.619
500	1.255 1.282	1.269	1.232
1000	2.400 2.411	2.406	2.369

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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. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE/CELL LYSATE/SALIVA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (pg/mL)	95.0	193	391	96.0	191	388
Standard deviation	4.26	8.81	24.3	8.30	14.8	22.2
CV (%)	4.5	4.6	6.2	8.6	7.7	5.7

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)	102	203	408	102	202	407
Standard deviation	3.45	5.73	23.0	7.59	12.2	17.8
CV (%)	3.4	2.8	5.6	7.4	6.0	4.4

RECOVERY

The recovery of human TRAIL spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	101	92-105%
Serum (n=4)	96	91-102%
EDTA plasma (n=4)	103	96-112%
Heparin plasma (n=4)	103	98-115%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human TRAIL were serially diluted with the appropriate calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Cell lysates* (n=2)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva* (n=4)
1:2	Average % of Expected	105	95	107	106	104	105
	Range (%)	102-107	92-98	104-113	101-110	100-112	101-112
1:4	Average % of Expected	104	95	105	109	105	102
	Range (%)	100-108	91-100	101-108	106-113	95-113	88-114
1:8	Average % of Expected	101	97	109	106	102	112
	Range (%)	95-105	93-100	102-116	103-111	99-105	110-115
1:16	Average % of Expected	102	94	104	107	97	114
	Range (%)	96-110	89-98	99-111	101-111	85-104	110-118

*Samples were diluted/lysed prior to assay.

SENSITIVITY

Seventy-eight assays were evaluated and the minimum detectable dose (MDD) of human TRAIL ranged from 0.57-7.87 pg/mL. The mean MDD was 2.86 pg/mL.

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

CALIBRATION

This immunoassay is calibrated against a highly purified recombinant human TRAIL produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Saliva - Samples from apparently healthy volunteers were evaluated for the presence of human TRAIL 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=60)	76	28-135	25
EDTA plasma (n=35)	82	34-163	27
Heparin plasma (n=35)	88	37-151	30
Saliva (n=7)	472	177-1302	392

Cell Culture Supernates/Cell Lysates:
Human peripheral blood mononuclear cells (5 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. 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 human TRAIL.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated cells	ND	ND
Stimulated cells	ND	38.8

ND-Non-detectable

N1186 human T cells were cultured in RPMI supplemented with 10% fetal bovine serum and 10 ng/mL of recombinant human IL-2. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 3 days. Aliquots of the cell culture supernates and cell lysates were removed and assayed for levels of human TRAIL.

Condition	Cell culture supernates (pg/mL)	Cell Lysates (pg/mL)
Unstimulated cells	34.0	783
Stimulated cells	33.3	705

SPECIFICITY

This assay recognizes natural and recombinant human TRAIL.

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 recombinant human TRAIL control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

4-1BB Ligand
APRIL
BAFF/BLyS
CD27 Ligand
CD30 Ligand
CD40 Ligand
Fas Ligand
GITR Ligand
LIGHT
LT α_1/β_2
LT α_2/β_1
OPG
OX40 Ligand
TNF-α
TNF-β
TRAIL R3
TRAIL R4
TRANCE
TWEAK
VEGI

Recombinant mouse:

CD27 Ligand
CD30 Ligand
Fas Ligand
LT α_1/β_2
LT α_2/β_1
OX40 Ligand
TNF-α
TRANCE

Other recombinants:

porcine TNF-α
rat TNF-α

Recombinant human TRAIL R1 interferes at concentrations > 10 ng/mL.

Recombinant human TRAIL R2 interferes at concentrations > 25 ng/mL.

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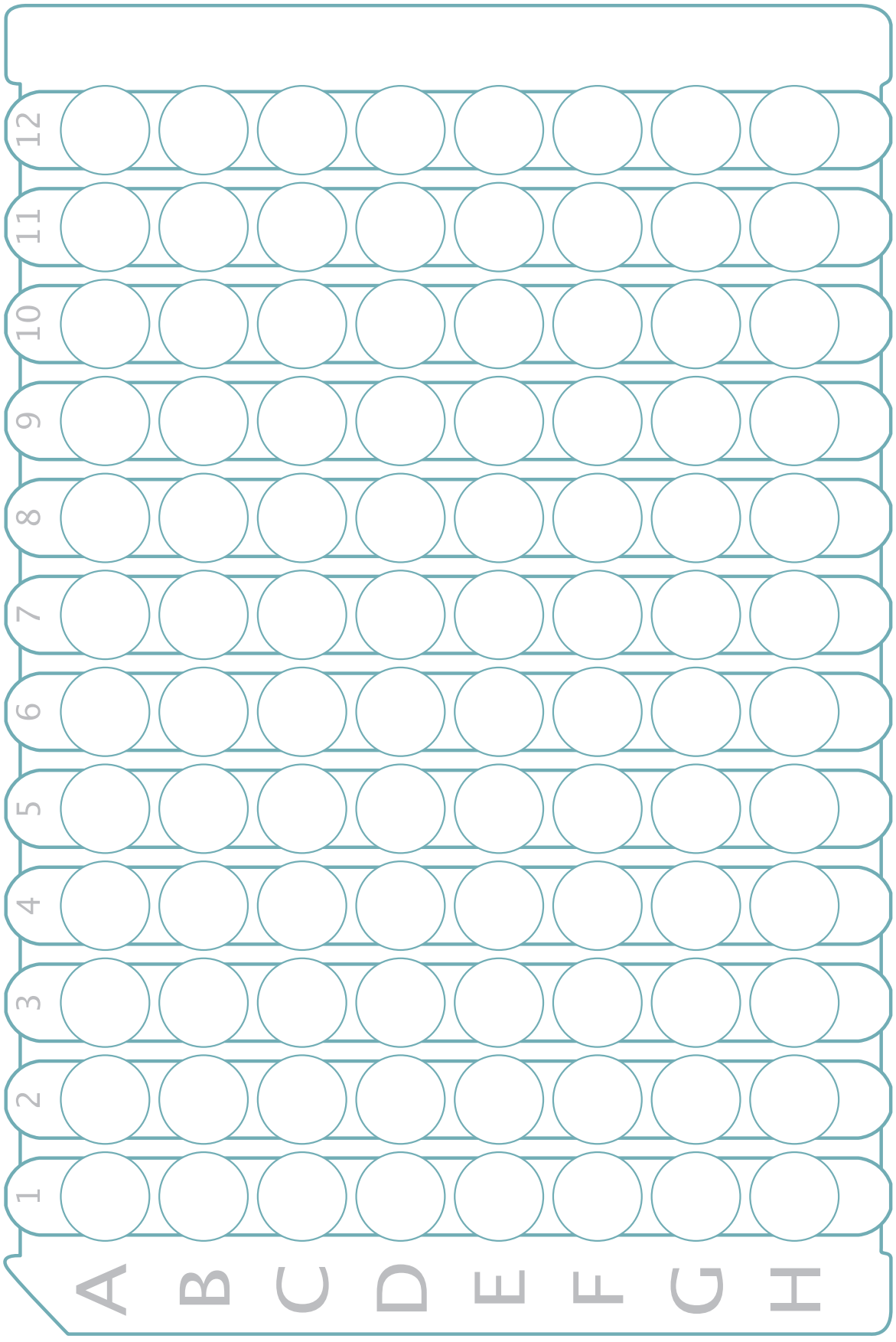
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES

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