KAMIYA BIOMEDICAL COMPANY

sTRAIL ELISA

For the detection and analysis of Tumor Necrosis Factor-related apoptosis inducing ligand (TRAIL) in lysates and fluid samples

Cat. No. KT-333

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

PRODUCT INFORMATION

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INTENDED USE
The sTRAIL ELISA is for the detection and analysis of Tumor Necrosis Factor-related apoptosis inducing ligand (TRAIL) in lysates and fluid samples. The kit is suitable for use with human samples. For research use only. Not for use in diagnostic procedures.

INTRODUCTION
Tumor necrosis factor-related apoptosis inducing ligand (TRAIL)/Apo2L is a type II transmembrane protein that was identified and cloned based on its sequence homology with members of the TNF ligand family. Both the transmembrane and soluble recombinant forms of TRAIL can rapidly induce apoptosis in a wide variety of transformed human cell lines *in vitro*, yet no cytotoxicity is observed with normal human cell lines, even though TRAIL and its receptors are expressed in both cell types. TRAIL is expressed constitutively in many tissues such as liver, lung, placenta, kidney, spleen, peripheral lymphocyte, etc. It is highly expressed in cancer cells and selectively kills the cancer cells by binding two cell-surface receptors, DR4 and DR5, which are highly expressed in cancer cells. TRAIL induces cancer apoptosis independently of p53 gene mutation. Two additional TRAIL decoy receptors (DcR1 and DcR2) do not trigger an apoptotic signal in normal cells and have been proposed to confer protection from TRAIL-inducing apoptosis. TRAIL may also play roles in immune responses, autoimmune disorders and HIV infections. TRAIL can interact with five different members of the TNF receptor superfamily, two of which contain a death domain, and can mediate an apoptotic response. TRAIL may also be a marker for progression of cancer.

PRINCIPLE
The sTRAIL ELISA is a useful tool to capture and quantifiably measure the amount of TRAIL in a sample. The Sandwich ELISA method for detecting a protein in a sample uses two antibodies that each recognizes distinct epitopes on the protein. An ELISA plate is coated with the first antibody, called the capture antibody, which is used to capture the protein from the sample. The second antibody, called the Detecting Antibody, is used to detect the protein bound to the Capture Antibody. The Capture Antibody recognizes one epitope while the polyclonal Detecting Antibody recognizes many. An alkaline phosphatase (AP)-conjugated secondary antibody is then used for colorimetric detection. The sTRAIL ELISA measures the soluble form of TRAIL (sTRAIL). It rapidly provides quantifiable results using a calibration curve that is easily generated using the included recombinant sTRAIL. The kit can detect picogram quantities of sTRAIL in lysates or fluid samples.

ADVANTAGES
- Multiple samples can be analyzed in low-volume, high-throughput experiments
- Analysis is complete in just hours
- Quantitative

COMPONENTS

Reagents	Quantity	Composition
• Detecting Antibody	10 µL	0.25 mg/mL in PBS and 0.1% sodium azide.
• Conjugate Solution	10 µL	alkaline phosphatase conjugated secondary antibody in PBS and 0.1% sodium azide.
• Lysis Buffer	10 mL	in 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.4% NP-40, 1 mM EDTA, 1 mM EGTA, 20 µM sodium orthovanadate
• Protease Inhibitor Cocktail	100 µL	concentrate (100X)
• BSA Powder	0.3 g	dilute with Wash Buffer to make Blocking Buffer
• sTRAIL Calibrator	500 µL	Recombinant sTRAIL, concentration 20 ng/mL

- Wash Buffer 25 mL concentrate (20X) PBS, pH 7.4, 1% Tween 20, 4 mM sodium orthovanadate
- Substrate Solution 10 mL pNPP solution
- Microtiter Plate 96 wells 12 X 8 microwell strips
- Plate Sealer 1

Note: The included buffers and reagents have been optimized for use with this kit. Substitutions with other reagents may not give optimal results.

Materials Required But Not Provided

- Distilled water (dH_2O)
- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Microplate spectrophotometer
- Orbital shaker
- Phosphate buffered saline (PBS, pH 7.4)

STORAGE

The kit should be kept at 4°C with the exception of the Protease Inhibitor Cocktail and sTRAIL Calibrator which should be kept at -20°C.

PRECAUTIONS

1. Read the instructions carefully before beginning the assay.
2. This kit is for research use only, not for human or diagnostic use.
3. Great care has been taken to ensure the quality and reliability of this product. However, it is possible that in certain cases, unusual results may be obtained due to high levels of interfering factors.

PROTOCOLS

Preparation of Reagents

Complete Lysis Buffer: An excess of Lysis Buffer is provided for preparing cell extracts for the assay. Prepare the amount of Complete Lysis Buffer required for the assay by adding 10 µL of Protease Inhibitor Cocktail per mL of Lysis Buffer.

Note: Some protease inhibitors lose activity within 24 hours of dilution. Therefore, we recommend using the Complete Lysis Buffer immediately for cell lysis. The remaining amount should be discarded if not used in the same day.

Wash Buffer: 1X Wash Buffer may be stored at 4°C. If you do not plan on using the entire ELISA plate at once, we recommend preparing only enough Wash Buffer to meet the needs of the experiment.

Dilute 20X Wash Buffer to 1X with dH_2O. The following chart can be used as a guide.

For 1X Wash Buffer	20X Wash Buffer	dH2O	Sufficient for # reactions
25 mL	1.25 mL	23.75 mL	5
50 mL	2.5 mL	47.5 mL	10
100 mL	5 mL	95 mL	20
250 mL	12.5 mL	237.5 mL	48
500 mL	25 mL	475 mL	96

Blocking Buffer: Dissolve 0.3 g BSA in 30 mL of 1X Wash Buffer in a sterile bottle.

Note: To prevent microbial growth, store Blocking Buffer at 4°C and reduce exposure to contaminants. Dissolve BSA in 1X Wash Buffer only when ready to use.

Preparation of Whole-Cell Extracts

This procedure can be used for a confluent cell layer of 25 cm^2 (60 mm dish). The yield is approximately 0.3 mg of total cellular protein.

1. Treat the cells as required for activation.
2. Wash the cells 2X with 8 mL ice-cold PBS (10 mM phosphate buffer, pH 7.4, 150 mM NaCl).
3. Collect the cells in 3 mL ice cold PBS. Use a cell scraper for adherent cells.
4. Centrifuge the cells for 10 minutes at 1,000 rpm at 4°C.
5. Discard the supernatant and add a volume of Complete Lysis Buffer equivalent to the volume of the cell pellet. Pipette gently to mix.
6. Transfer into a 1.5 mL microcentrifuge tube.
7. Incubate on ice for 30 minutes.
8. Centrifuge for 20 minutes at 14,000 X g (full speed in a microcentrifuge) at 4°C.
9. Collect the supernatant at 4°C, aliquot and store at -80°C. Avoid freeze/thaw cycles.
10. Measure the protein content by a Bradford-based assay.

sTRAIL ASSAY

Determine the appropriate number of microwell strips required for testing your samples, controls and blanks in duplicate. If less than 8 wells in a strip are to be used, cover the unused wells with a portion of the plate sealer before performing the assay. The contents of these wells are stable at room temperature and, if kept dry, can be used later for a separate assay. Store the unused strips in the aluminum pouch at 4°C. Use the strip holder for the assay. Prepare the Wash and Blocking Buffers as described above. Multi-channel pipettor reservoirs may be used for dispensing the various buffers and Substrate Solutions into the wells being used.

Calibration Curve Preparation

1. Set up a TRAIL Calibration Curve in duplicate using the following concentrations: 20.0, 10.0, 5.0, 2.5, 1.25, 0.63, 0.32 and 0.0 ng/mL.
   Note: for higher sensitivity, set up one or both TRAIL calibration curves using a 10-fold dilution of the sTRAIL Calibrator in Blocking Buffer (final concentration of 2.0, 1.0, 0.5, 0.25, 0.063, 0.032, 0.0 ng/mL).
2. Add 100 µL of Blocking Buffer to wells B1-H1 and B2-H2.
3. Pipette 100 µL sTRAIL Calibrator solution (20 ng/mL) into wells A1, A2, B1 and B2.
4. Mix wells B1 and B2 by pipetting
5. Transfer 100 µL from well B1 to C1 and B2 to C2.
6. Mix wells C1 and C2 by pipetting.
7. Transfer 100 µL from well C1 to D1 and C2 to D2
8. Continue this procedure to wells G1 and G2. After mixing, discard 100 µL of solution from wells G1 and G2.
9. Wells H1 and H2 are blanks and should only contain 100 µL of Blocking Buffer.

Binding of sTRAIL

1. Sample wells: dilute concentrated cell lysates to 1 mg/mL in Blocking Buffer. Pipette 100 µL lysate into each well.
2. Cover the plate with the plate sealer and incubate at room temperature for 4 hours.
3. Remove the cell lysate from wells and wash four times (4X) with 250 µL of Wash Buffer. After final wash, tap plate upside down several times to remove residual Wash Buffer.

Binding of TRAIL Detecting Antibody

1. Dilute Detecting Antibody 1:500 in Blocking Buffer. For one entire plate use 10 µL Detecting Antibody in 5 mL of Blocking Buffer.
2. Add 50 µL diluted Detecting Antibody to each well.
3. Cover the plate and incubate at room temperature for 1 hour.
4. Remove Detecting Antibody and wash wells four times (4X) with 250 µL Wash Buffer. After final wash, tap plate upside down several times to remove residual Wash Buffer.

Binding of Secondary Antibody

1. Dilute Secondary Antibody 1:1,000 in Blocking Buffer. For one entire plate, use 10 µL Secondary Antibody in 10 mL of Blocking Buffer.
2. Add 100 µL diluted Secondary Antibody to each well.
3. Cover the plate and incubate at room temperature for 1 hour.
4. Remove Secondary Antibody and wash thoroughly five times (5X) with 250 µL Wash Buffer to remove any unbound Secondary Antibody. Let plate sit for 1 minute and then tap plate upside down several times to remove residual Wash Buffer.

Colorimetric Determination

1. Add 100 µL Substrate Solution to each well.
2. Cover the plate and incubate at room temperature for 60 minutes.

   Note: Incubation time may need to be varied depending on the TRAIL concentration in the samples. While 60 minutes is suggested, optimal incubation times may need to be empirically determined. Calibration curves in the 20.0-0.32 ng/mL range develop faster than the 2.0-0.032 ng/mL range.
3. Read plate at 405 nm using an appropriate 96-well spectrophotometer.

TROUBLESHOOTING

Problem	Possible Cause	Recommendation
No signal or weak signal in all wells	Omission of key reagent	Check that all reagents have been added in the correct order.
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity.
	Enzyme inhibitor present	Inorganic phosphate (Pi), monoethanolamine, Be^{2+}, chelators of divalent metal ions (EDTA, oxalate, citrate, cysteine, histidine), acid or neutral pH, aromatic amino acids (Phe, Trp), L-homo-arginine, urea, iodoacetamide and high levels of Zn^{2+} are inhibitory to alkaline phosphatase.
	Plate reader settings not optimal	Verify the wavelength and filter settings of the plate reader.
	Incorrect assay temperature	Bring substrate to room temperature.
	Inadequate volume of Substrate Solution	Check to make sure that correct volume is delivered by pipette.
High background in all wells	Concentration of antibodies too high	Increase antibody dilution.
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations.
Uneven signal development	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations.
	Well cross-contamination	Follow washing recommendations.
High background in sample wells	Too much cell extract per well	Decrease amount of cell extract.
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start by using a 1:2,000 dilution for Detecting Antibody and a 1:2,000 dilution for the Secondary Antibody. The sensitivity of the assay will decrease.
No signal or weak signal in sample wells only	Not enough cell extract per well	Increase amount of cell extract.
	TRAIL is poorly induced	Perform a time course for TRAIL induction in the studied cell line.
	Cell extracts are not from human origin	Perform study with a human model.

FOR RESEARCH USE ONLY

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