Version 24-05/24

**User's Manual**

# TRAIL R4 (Human) ELISA Kit

REF **DEIA4330**

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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# PRODUCT INFORMATION

## Intended Use

The TRAIL R4 (Human) ELISA Kit is a solid phase sandwich ELISA for the in-vitro qualitative and quantitative determination of TRAIL R4/DcR2 in cell culture supernatants, buffered solutions or human serum, plasma, or other body fluids. This assay will recognize both natural and recombinant human TRAIL R4/DcR2. This kit has been configured for research use only.

## General Description

Human Trail R4, also called DcR1, CD264 or TRUNND is a type 1 TNF R family membrane protein which is a receptor for TRAIL. In the Trail receptor family, Trail R4 (DcR2) but also Trail R3 (DcR1) antagonize TRAIL-induced apoptosis whereas Trail R1 (DR4) and Trail R2 (DR5) transducer an apoptosis signal. Trail R4 has a truncated intracellular domain. Overexpression of Trail R4 has been shown to protect cells bearing Trail R1 and/or Trail R2 from Trail-induced apoptosis. Many tumor cell lines don't express the decoy receptors and are therefore sensitive to TRAIL if they express Trail R1 and/or Trail R2.

## Principles of Testing

A capture Antibody highly specific for TRAIL R4 has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of TRAIL R4 in samples and known standards to the capture antibodies is completed and then any excess unbound analyte is removed. During the next incubation period the binding of the biotinylated anti- TRAIL R4 secondary antibody to the analyte occurs. Any excess unbound secondary antibody is then removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of TRAIL R4 present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of TRAIL R4 in any sample tested.

## Reagents And Materials Provided

1. 96 well microtiter strip plate: Ready to use (Pre-coated), 1 plate
2. Plastic plate covers: 2 pieces
3. Standard: 10,000 pg/mL. Reconstitute as directed on the vial, 2 vials
4. Standard Diluent (Buffer): 10× Concentrate, dilute in distilled water, 25 mL
5. Biotinylated anti-TRAIL R4: Dilute in Biotinylated Antibody Diluent, 0.4 mL
6. Biotinylated Antibody Diluent: Ready to use, 7 mL
7. Streptavidin-HRP: Add 0.5 mL of HRP diluent prior to use, 5 µL × 2

8. HRP Diluent: Ready to use, 23 mL
9. Wash Buffer: 200× Concentrate dilute in distilled water 10 mL
10. TMB Substrate: Ready to use, 11 mL
11. $H_2SO_4$ stop reagent: Ready to use, 11 mL

## Materials Required But Not Supplied

1. Microtiter plate reader fitted with appropriate filters (450 nm required with optional 630 nm reference filter)
2. Microplate washer or wash bottle
3. 10, 50, 100, 200 and 1,000 µL adjustable single channel micropipettes with disposable tips
4. 50-300 µL multi-channel micropipette with disposable tips
5. Multichannel micropipette reagent reservoirs
6. Distilled water
7. Vortex mixer
8. Miscellaneous laboratory plastic and/or glass, if possible sterile

## Storage

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the kit and reagents is stated on box front labels. The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

Wash Buffer: Once prepared store at 2-8°C for up to 1 week

Standard Diluent Buffer: Once prepared store at 2-8°C for up to 1 week

Standards : Once prepared use immediately and do not store

Biotinylated Secondary Antibody: Once prepared use immediately and do not store

Streptavidin-HRP: Once prepared use immediately and do not store

## Specimen Collection And Preparation

Cell culture supernatants, serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 × g for 10 min.

Serum: Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 × g for 10 min and remove serum.

Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 × g for 30 min to remove.

Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500 µL) to avoid

repeated freeze-thaw cycles and stored frozen at -70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

## Plate Preparation

| | Standards | | Sample Wells | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 10000 | 10000 | | | | | | | | | | |
| B | 5000 | 5000 | | | | | | | | | | |
| C | 2500 | 2500 | | | | | | | | | | |
| D | 1250 | 1250 | | | | | | | | | | |
| E | 625 | 625 | | | | | | | | | | |
| F | 312.5 | 312.5 | | | | | | | | | | |
| G | Blank | Blank | | | | | | | | | | |
| H | | | | | | | | | | | | |

## Reagent Preparation

Bring all reagents to room temperature before use

1. Assay Design: Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested in duplicate. Remove sufficient microwell strips for testing from the pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C storage.
2. Preparation of Wash Buffer: Dilute the (200×) wash buffer concentrate 200 fold with distilled water to give a 1× working solution. Pour entire contents (10 mL) of the Washing Buffer Concentrate into a clean 2,000 mL graduated cylinder. Bring final volume to 2,000 mL with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2-8˚C for up to 1 week.
3. Preparation of Standard Diluent Buffer: Add the contents of the vial (10× concentrate) to 225 mL of distilled water before use. This solution can be stored at 2-8°C for up to 1 week.
4. Preparation of Standard: Standard vials must be reconstituted with the volume of standard diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 10,000 pg/mL of TRAIL R4. Mix the reconstituted standard gently by inversion only. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 10,000 to 312.5 pg/mL. A fresh standard curve should be produced for each new assay.

Immediately after reconstitution add 200 µL of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 10000 pg/mL

Add 100 µL of Standard Diluent to the remaining standard wells B1 and B2 to F1 and F2

Transfer 100 µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells

Continue this 1:1 dilution using 100 µL from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 1000 pg/mL to 31.25 pg/mL

Discard 100 µL from the final wells of the standard curve (F1 and F2)

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

5. Preparation of Samples: Before testing, human serum or plasmas samples have to be diluted 1:4 in standard buffer diluent.
6. Preparation of Biotinylated Anti TRAIL R4 It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti TRAIL R3 with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

| Number of wells required | Biotinylated Antibody (µL) | Biotinylated Antibody Diluent (µL) |
|---|---|---|
| 16 | 40 | 1060 |
| 24 | 60 | 1590 |
| 32 | 80 | 2120 |
| 48 | 120 | 3180 |
| 96 | 240 | 6360 |

7. Preparation of HRP-Conjugate: It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom. Dilute the 5 µL vial with 0.5 mL of HRP diluent immediately before use. Do-not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

| Number of wells required | Streptavidin-HRP (µL) | Streptavidin-HRP Diluent (mL) |
|---|---|---|
| 16 | 30 | 2 |
| 24 | 45 | 3 |
| 32 | 60 | 4 |
| 48 | 75 | 5 |
| 96 | 150 | 10 |

## Assay Procedure

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only. Prepare all reagents as shown in section Reagent Preparation.

Note: Final preparation of Biotinylated anti TRAIL R4 and Streptavidin-HRP should occur immediately before use.

1. Addition: Prepare Standard curve as shown in section Reagent Preparation.
2. Addition: Add 100 µL of Standard Diluent to zero wells and 100 µL of Samples to designated wells.
3. Incubation: Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 2 hour(s).

4. Wash: Remove the cover and wash the plate as follows:
   Aspirate the liquid from each well
   Dispense 0.3 mL of 1× washing solution into each well
   Aspirate the contents of each well
   Repeat step b and c another two times
5. Addition: Add 50 µL of diluted biotinylated anti TRAIL R4 to all wells.
6. Incubation: Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 1 hour.
7. Wash: Repeat wash step 4.
8. Addition: Add 100 µL of Streptavidin-HRP solution into all wells.
9. Incubation: Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 30 min.
10. Wash: Repeat wash step 4.
11. Addition: Add 100 µL of ready-to-use TMB Substrate Solution into all wells.
12. Incubation: Incubate in the dark for 10-20 minutes* at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
13. Addition: Add 100 µL of $H_2SO_4$:Stop Reagent into all wells.
14. Read the absorbance value of each well (immediately after step 13.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

*Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range.

## Calculation

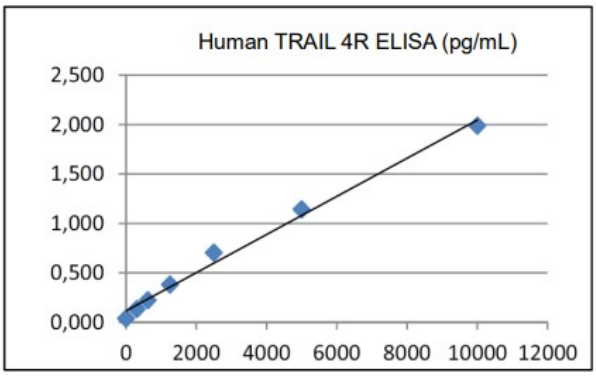
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding human TRAIL R4 standard concentration on the horizontal axis.

The amount of TRAIL R4 in each sample is determined by extrapolating OD values against TRAIL R4 standard concentrations using the standard curve.

## Typical Standard Curve

| Standard | TRAIL R4 Conc | OD (450 nm) mean | CV (%) |
|---|---|---|---|
| 1 | 10000 | 1.989 | 0.6 |
| 2 | 5000 | 1.142 | 0.7 |
| 3 | 2500 | 0.705 | 7.3 |
| 4 | 1250 | 0.381 | 1.3 |
| 5 | 625 | 0.224 | 1.7 |
| 6 | 312.5 | 0.134 | 9.5 |
| Zero | 0 | 0.039 | - |




Note: curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

For serum and plasma samples which have been diluted according to the protocol (1:4), the calculated concentration should be multiplied by the dilution factor (×4).

## Precision

| Intra-Assay | | | | | Inter-assay | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample | n | Mean (pg/mL) | SD | CV% | Sample | n | Mean (pg/mL) | SD | CV% |
| A | 8 | 5723 | 212 | 3.7 | A | 12 | 6531 | 627 | 9.6 |
| B | 8 | 3063 | 218 | 7.1 | B | 12 | 3123 | 369 | 11.8 |

## Sensitivity

The sensitivity, minimum detectable dose of human TRAIL R4 using this TRAIL R4 (Human) ELISA Kit was found to be 64 pg/mL. This was determined by adding 3 standard deviations to the mean OD obtained when the zero standard was assayed 32 times.

## Specificity

The assay recognizes natural and recombinant human TRAIL R4. To define specificity of this ELISA, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested: TRAIL, CD117, IL-6R, IL-2R, CD116, TRAIL R2, TRAIL R3, TRAIL R1, CD178 and Granzyme B.

## Linearity

One human serum containing 10,000 pg/mL of TRAIL R4 was serially diluted in standard buffer diluent. Linear regression of samples versus the expected concentration yielded a correlation coefficient of 0.99.

## Recovery

The spike recovery was evaluated by spiking concentrations of natural TRAIL R4 in human serum. Recoveries ranged from 68% to 87% with an overall mean recovery of 77%.

## Precautions

1. Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984
2. Laboratory gloves should be worn at all times
3. Avoid any skin contact with $H_2SO_4$ and TMB. In case of contact, wash thoroughly with water
4. Do not eat, drink, smoke or apply cosmetics where kit reagents are used
5. Do not pipette by mouth
6. When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels
7. All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use
8. Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration
9. Cover or cap all reagents when not in use
10. Do not mix or interchange reagents between different lots
11. Do not use reagents beyond the expiration date of the kit
12. Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of $H_2SO_4$ and substrate solution, avoid pipettes with metal parts
13. Use a clean plastic container to prepare the washing solution
14. Thoroughly mix the reagents and samples before use by agitation or swirling
15. All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells
16. The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly
17. If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance's within 1 hour after completion of the assay
18. When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells
19. Follow incubation times described in the assay procedure
20. Dispense the TMB solution within 15 min of the washing of the microtitre plate

21. Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.
22. The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.
23. Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
24. Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
25. As with most biological assays conditions may vary from assay to assay therefore a fresh standard curve must be prepared and run for every assay.