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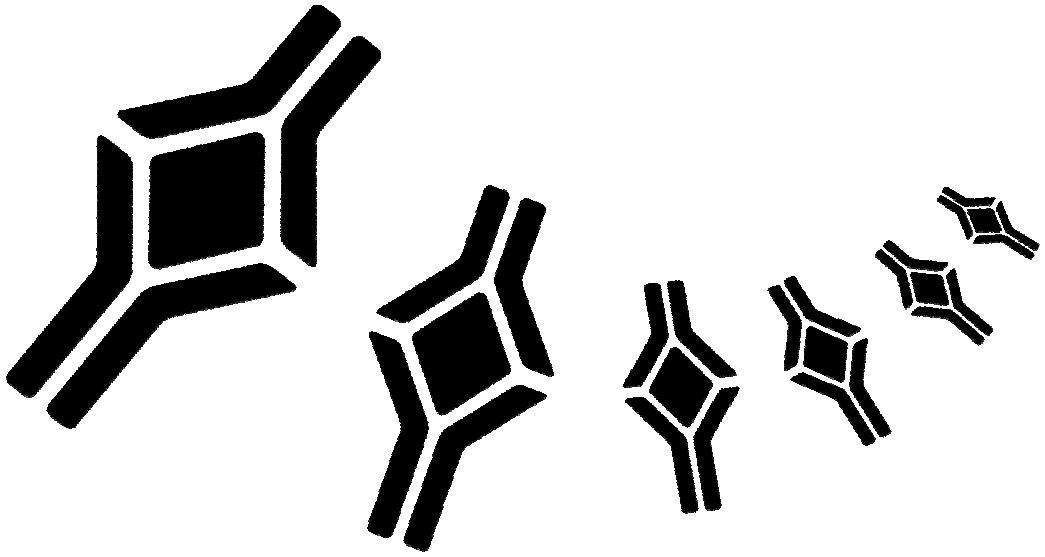


Human Thioredoxin Reductase ELISA

Product Data Sheet

Cat. No.: RLF-EK0122R

For Research Use Only

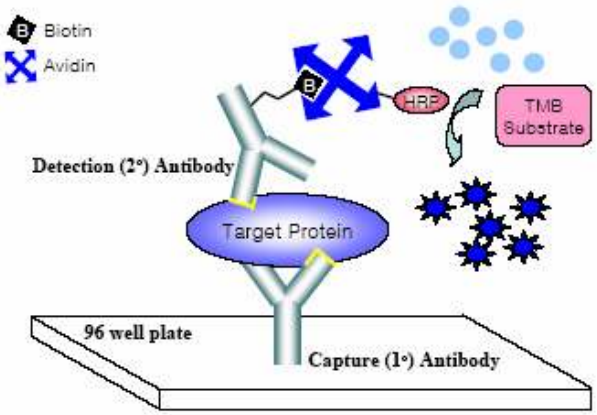
VERSION 51 160708

1. INTRODUCTION

The mammalian thioredoxin reductases(TrxRs) are a family of selenocysteine-containing pyridine nucleotide-disulfide oxido-reductases. Three different TrxR isoenzymes, TrxR1 as cytosolic, TrxR2 as mitochondrial, and TrxR3 as testis-specific thiol regulator are known. All the mammalian TrxRs are homologous to glutathione reductase with respect to primary structure including the conserved redox catalytic site (-Cys-Val-Asn-Val-Gly-Cys-) but distinctively with a C-terminal extension containing a catalytically active penultimate selenocysteine(SeCys) residue in the conserved sequence(-Gly-Cys-SeCys-Gly). TrxR is homodimeric protein in which each monomer includes an FAD prosthetic group, a NADPH binding site and a redox catalytic site. Electrons are transferred from NADPH via FAD and the active-site disulfide to C-terminal SeCys-containing redox center, which then reduces the substrate like thioredoxin. The members of TrxR family are 55–58 kDa in molecular size and composed of three isoforms including cytosolic TrxR1, mitochondrial TrxR2, and TrxR3, known as Trx and GSSG reductase(TGR). TrxR plays a key role in protection of cells against oxidative stress and redox-regulatory mechanism of transcription factors and various biological phenomena. Many tumor cells have elevated TrxR levels and TrxR has been shown to play a major role in drug resistance. Inhibition of TrxR and its related redox reactions may thus contribute to a successful single, combinatory or adjuvant cancer therapy. A great number of effective natural and synthetic TrxR inhibitors are now available possessing antitumor potential ranging from induction of oxidative stress to cell cycle arrest and apoptosis.

2. PRINCIPLES OF METHOD

The design of this assay is based on a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to human TR1. Samples are pippetted into these wells. Unbound TR1 and other components of the sample are removed by washing, then biotin-conjugated monoclonal antibody specific to TR1 is added. In order to quantitatively determine the amount of TR1 present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) is then added to each microplate well. Next, a TMB-substrate solution is added to each well. Finally, sulfuric acid solution is added and the resulting yellow colored product is measured at 450nm. The absorbance (O.D. value) is directly proportional to the amount of captured TR1.



Sandwich ELISA method

3. INTENDED USE

The human Thioredoxin Reductase-1 (human TR1) ELISA kit is to be used for the in vitro quantitative determination of human TR1 in human serum, human plasma, cell lysate and buffered solution. The assay will recognize both native and recombinant human TR1.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

4. STORAGE AND STABILITY

All components of this kit are stable at 2 to 8°C. Any unused reconstituted standard should be discarded or frozen at -70°C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

5. CHEMICAL HAZARD

- Stop solution: This reagent is an irritant to eyes, skin and mucous m embranes. Avoid contact with eyes, skin and clothing. Wear suitable protec tive clothing, gloves and eye protection. In the event of contact with eyes or skin, wash immediately with plenty of water.

- All reagents containing Sodium Azide also contain Thimerosal as a pr eservative. Thimerosal contains Hg thus should be handled with great care.

6. KIT CONTENTS

Contents	Number	Volume
96 Well Plate	1 (in aluminum foil bag with desiccant)	
Incubation Buffer	1	30ml
Washing Buffer	1	(10X) 100ml
Standard Protein	1	
+Standard/Sample Dilution Buffer	1	25ml
Secondary Antibody	1	(100X) 150µl
AV-HRP	1	(100X) 150µl
Secondary Antibody/AV-HRP Dilution Buffer	1	25ml
Substrate (TMB)	1	20ml
Stop Solution	1	20ml
Protocol booklet	1	
Plate sealers	3	

① 96 Well Plate

: Human TR1 microtiter plate, one plate of 96 wells (16well strip x 6).
A plate using break-apart strips coated with a mouse monoclonal antibody specific to human TR1.

② Standard Protein

: Recombinant human TR1.

③ Secondary Antibody

: Biotin labeled mouse anti human TR1 antibody.

④ AV-HRP

: Avidin linked Horseradish Peroxidase (HRP, enzyme)

⑤ Substrate (Stabilized chromogen)

: Tetramethylbenzidine(TMB) solution

⑥ Stop Solution

: 1N solution of sulfuric acid (H_2SO_4).

⑦ Plate sealer

: Adhesive sheet.

- Do not mix or interchange reagents from different lots.

7. MATERIALS REQUIRED BUT NOT PROVIDED

① Microtiter plate reader capable of measurement at or near 450nm.

② Calibrated, adjustable precision pipettes, preferably with disposable plast c tips
(A manifold multi-chanel pipette is desirable for large assays.)

③ Distilled or deionized water

④ Data analysis and graphing software

⑤ Vortex mixer

⑥ Polypropylene tubes for diluting and aliquoting standard

⑦ Absorbent paper towels

⑧ Calibrated beakers and graduated cylinders of various sizes

8. REAGENT PREPARATION

1) Human TR1 standard

Reconstitute the human TR1 standard to 250 ng/ml by adding 1ml of *Standard/Sample Dilution Buffer* into the standard protein glass vial containing lyophilized human TR1 protein. Swirl or mix gently, and allow to sit for 5 minutes to ensure complete reconstitution.

	Standard	**Add**	**Into**
#1	25.00ng/ml	100 µl l of the 500ng/ml standard	900 µl of the *Standard/Sample Dilution Buffer*
#2	12.50ng/ml	500 µl of tube #1	500 µl of the *Standard/Sample Dilution Buffer*
#3	6.25ng/ml	500 µl of tube #2	500 µl l of the *Standard/Sample Dilution Buffer*
#4	3.13ng/ml	500 µl of tube #3	500 µl of the *Standard/Sample Dilution Buffer*
#5	1.56ng/ml	500 µl of tube #4	500 µl of the *Standard/Sample Dilution Buffer*
#6	0.78ng/ml	500 µl of tube #5	500 µl of the *Standard/Sample Dilution Buffer*
#7	0.39ng/ml	500 µl of tube #6	500 µl of the *Standard/Sample Dilution Buffer*
#8	0ng/ml	1.0 ml of the *Standard/Sample Dilution Buffer*	

2) Secondary Antibody

1. Equilibrate to room temperature, mix gently.
2. Mix 20 µl *Secondary Antibody concentrated solution* (100X) + 2ml *Secondary Antibody/AV-HRP dilution buffer*. (Sufficient for one 16-well strip, prepare more if needed) Label as “Working Secondary antibody Solution”.
3. Return the unused *Secondary Antibody concentrated solution* to the refrigerator.

3) AV-HRP

1. Equilibrate to room temperature, mix gently.
2. Mix 20 µl *AV-HRP concentrated solution* (100X) + 2ml *Secondary Antibody/AV-HRP dilution buffer*. (Sufficient for one 16-well strip, prepare more as needed) Label as "Working AV-HRP Solution".
3. Return the unused *AV-HRP concentrated solution* to the refrigerator.

4) Washing buffer

1. Equilibrate to room temperature, mix to re-dissolve any precipitated salt.
2. Mix 1 volume *Wash buffer concentrate solution* (10X) + 9 volumes of deionized water. Label as "Working Washing Solution".
3. Store both the concentrated and the Working Washing Solution in the refrigerator.

*** Directions for washing**

1. Fill the wells with 300µl of "Working Washing Buffer".
Let soak for 1 to 3 minutes and then all residual wash-liquid must be drained from the wells by aspiration (taking care not to scratch the inside of the well) or decantation, followed by forceful tapping of the plate on absorbent paper. Never insert absorbent paper directly into the wells.
If using an automated washer, the operating instructions for washing equipment should be carefully followed.
2. Incomplete washing or residual washing buffer in wells will adversely affect the assay and render false results.
3. It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing to avoid strips coming free of the frame.

9. ASSAY PROCEDURE

- Allow all reagents to reach room temperature before use.

Gently mix all liquid reagents prior to use.

- All standards, controls and samples should be run in duplicate for confi rmation of reproducibility.
- A standard curve must be run with each assay.
- If particulate matter is present in the analyte, centrifuge or filter prior t o analysis.
- Maintain a consistent order of sample and reagent additions from well t o well.

This ensures equal incubation times for all wells.

1) Determine the number of 16-well strips needed for assay. Insert these in the frame(s) for current
   use Re-bag extra strips and frame. Refrigerate for further use).
2) Add 300µl of *Incubation buffer* to all wells and incubate the plate for 5 minutes at room temperature.
3) Thoroughly aspirate or decant the solution from the wells. Wash wells 2 times (See “Directions for washing”).
4) For the standard curve, add 100µl of the standard to the appropriate microtiter wells.
   Add 100µl of the *Standard/Sample Dilution Buffer* to zero wells.
5) Serum requires at least 30 fold dilution in the *Standard/Sample Dilutio n Buffer.* And add 100ul of diluted samples to each well.
6) Cover the plate with the plate cover and incubate for 2 hours at roo m temperature.
7) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
8) Pipette 100µl of “Working Secondary Antibody Solution” into each wel l.

9) Cover the plate with the plate cover and incubate for 1 hour at room temperature.
10) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
11) Add 100µl “Working AV-HRP Solution” to each well.
12) Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
13) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
14) Pour enough Substrate you need into a tube or reagent boat. Add 100µl of *Substrate* to each well. The liquid in the wells should begin to turn blue.
15) Incubate the plate at room temperature. Avoid exposing the microtiter plate to direct sunlight.

- Do not cover the plate with aluminum foil (or other metal), or color may develop.
  The incubation time for chromogen substrate is often determined by the microtiter plate reader used. O.D. values should be monitored and the substrate reaction stopped before O.D. of the positive wells exceeds the limits of the instrument. O.D. values at 450nm can only be read after the Stop Solution has been added to each well.
- Keep the plate away from sun light because the *Substrate* is light se nsitive,.
- Typically, reaction is stopped 5~10 minutes after treatment of Substrat e, but this time can be adjusted as the user desires..

16) Add 100µl of *Stop Solution* to each well. The solution in the wells s hould change from blue to yellow.
17) Read the absorbance of each well at 450nm. Read the plate within 20 minutes of adding the *StopSolution*.

18) Plot on graph paper the absorbance of the standard against the stan dard concentration (Optimally,
the background absorbance can be subtracted from all data points, in cluding standards, unknowns and controls, prior to plotting.). Draw a s mooth curve through these points to construct the standard curve.
19) Read the human TR1 concentrations for the unknown samples and c ontrols from the standard curve plotted in step 18. Multiply value(s) o btained for the unknown sample by the dilution factor (Samples produ cing signals greater than that of the highest standard should be furth er diluted in the S*tandard/SampleDilution Buffer*).

10. CHARACTERISTICS

1) Typical result

The standard curve below is for illustration only and **should not be used** to calculate results in your assay.

A standard curve must be run with each assay.

Standard Human TR1 (ng/ml)	Optical Density (at 450nm)
0	0.061
0.39	0.080
0.78	0.097
1.56	0.145
3.13	0.217
6.25	0.375
12.50	0.701
25.00	1.400

11. LIMITATIONS

- Do not extrapolate the standard curve beyond the 25ng/ml standard poi nt.
- Other buffers and matrices have not been investigated.
- The rate of degradation of native human TR1 in various matrices has not been investigated.

2) Sensitivity

The minimal detectable dose of human TR1 was calculated to be 0.39 ng/ml, by subtracting two standard deviations from the mean of 10 zero standard replicates (ELISA buffer, S0) and intersecting this value with the standard curve obtained in the same calculation.

3) Specificity

The following substances have been were tested and found to have no cross-reactivity: human TR2.

4) Precision

① Within-Run (Intra-Assay)

(n=5)

Mean (ng/ml)	SD (ng/ml)	CV (%)
3.06	0.19	6.3
6.01	0.42	7.0
12.53	0.81	6.5
25.05	0.72	2.9

② Between-Run (Inter-Assay)

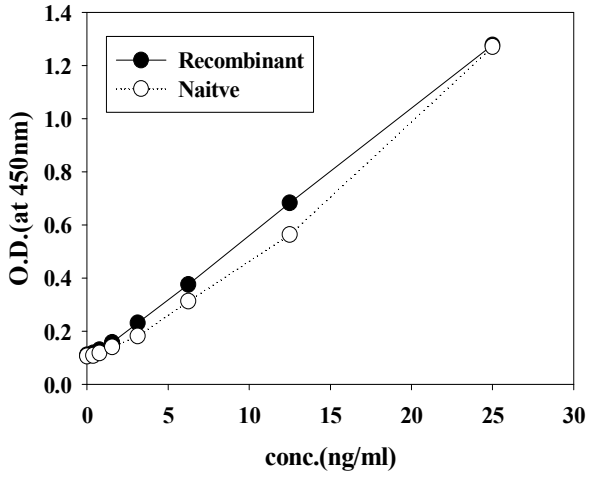
(n= 10)

Mean (ng/ml)	SD (ng/ml)	CV (%)
3.08	0.28	9.1
6.16	0.25	4.1
12.23	0.39	3.1
25.16	1.99	7.9

5) Recovery

Recovery upon addition is 97.3~101.0% (mean 98.6%)
Recovery upon dilution is 96.0~101.3% (mean 99.4%)

6) Linearity



Native human TR1 from HeLa cell lysate was serially diluted in sample dilution buffer. The optical density of each dilution was plotted against the recombinant human TR1 standard curve.

12. TROUBLESHOOTING

Problem	Possible Cause	Solution
High signal and background in all wells	• Insufficient washing	• Increase number of washes • Increase soaking time before aspiration/decanting step
	• Too much AV-HRP	• Check dilution, titration
	• Incubation time too long	• Reduce incubation time
	• Development time too long	• Decrease the incubation time before the stop solution is added
No signal	• Reagent added in incorrect order, or incorrectly prepared	• Review protocol
	• Standard has gone bad (If there is a signal in the sample wells)	• Check the condition of stored standard
	• Assay was conducted from an incorrect starting point	• Reagents allows to come to 20~30°C before performing assay
Too much signal – whole plate turned uniformly blue	• Insufficient washing – unbound AV-HRP remaining	• Increase number of washes carefully
	• Too much AV-HRP	• Check dilution
	• Plate sealer or reservoir reused, resulting in presence of residual AV-HRP	• Use fresh plate sealer and reagent reservoir for each step
Standard curve achieved but poor discrimination between point	• Plate not developed long enough	• Increase substrate solution incubation time
	• Improper calculation of standard curve dilution	• Check dilution, make new standard curve
No signal when a signal is expected, but standard	• Sample matrix is masking	• More diluted sample recommended

curve looks fine	detection	
Samples are reading too high, but standard curve is fine	• Samples contain protein levels above assay range	• Dilute samples and run again
Edge effect	• Uneven temperature around work surface	• Avoid incubating plate in areas where environmental conditions vary • Use plate sealer

13. REFERENCES

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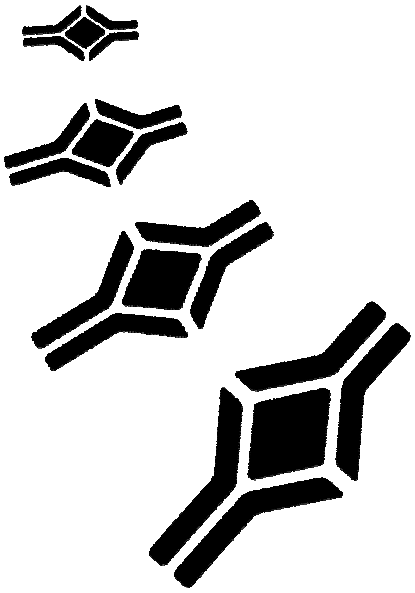

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