IMCO

The Expert in Thioredoxin and Glutaredoxin Systems

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FkTRX-02-V2

Kit for assay of thioredoxin

The thioredoxin system is the major protein disulfide reductase in cells and comprises thioredoxin, thioredoxin reductase and NADPH (1). Thioredoxin systems are ubiquitous (1-3) and hydrogen donors for ribonucleotide reductase, peroxiredoxins and methionine sulfoxide reductases. Thioredoxin systems are critical for cell viability, proliferation, activation of transcription factors and control of intracellular redox states. The activity of the thioredoxin system have been reported to be disrupted in various physiological disorders such as HIV infection, Alzheimer disease and diverse types of cancer and is therefore useful as a biological marker for several diseases (1-3). The classical way to determine the activity of the thioredoxin system is by insulin assays (4,5). This method is based on the reduction of insulin disulfides by reduced thioredoxin with thioredoxin reductase (6) and NADPH as ultimate electron donor. In this way, measurements of thioredoxin or thioredoxin reductase are possible by using a relative excess of the other component (7). Recently GSH and glutaredoxin have been shown to also reduce human Trx1 (8). The principle of the assay based on insulin labeled with fluorescein has been published (9). Note that in this assay (FkTRX-02-V2) eosin-labeled insulin is used (10) which has different wavelengths for excitation and emission as described below.
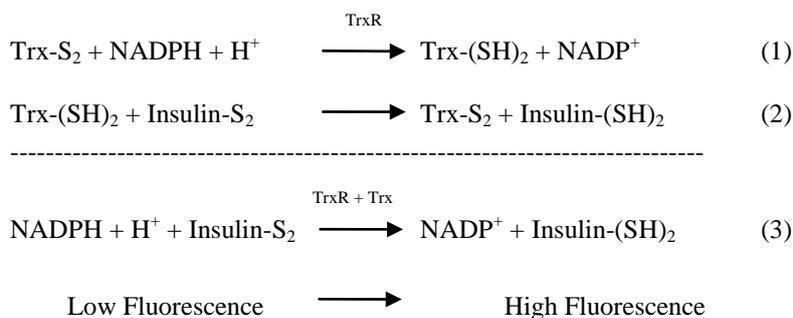
$$\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{\text{TrxR}} \text{Trx-(SH)}_2 + \text{NADP}^+ \qquad (1)$$

$$\text{Trx-(SH)}_2 + \text{Insulin-S}_2 \longrightarrow \text{Trx-S}_2 + \text{Insulin-(SH)}_2 \qquad (2)$$

$$\text{NADPH} + \text{H}^+ + \text{Insulin-S}_2 \xrightarrow{\text{TrxR + Trx}} \text{NADP}^+ + \text{Insulin-(SH)}_2 \qquad (3)$$

Low Fluorescence $\longrightarrow$ High Fluorescence

Fig 1. Scheme of reactions that form the basis of the FkTRX assays (9, 10).

BUFFERS, SOLUTIONS AND KIT CONTENT

Required for the assay

Fluorescence micro plate reader with 520 nm excitation 545 nm emission wavelengths (filters)

96 well black micro titer plates. Fluoresence plate reader like Perkin Elmer Enspire.

Supplied components

Assay Buffer

0.1 mg/ml Bovine serum albumin in 50 mM Tris-Cl and 1 mM EDTA, pH 7.5 (TE Buffer).

Preparation procedure:

Dissolve the lyophilized Assay buffer with 1.0 ml distilled water, transfer to a larger flask and add additionally 9.0 ml distilled water to a final volume of 10 ml.

Store frozen or at +4°C.

β-NADPH

Lyophilized reduced β-NADPH containing stabilizing reagents.

Preparation procedure:

Dissolve the content with 0.5 ml distilled water.

Store at -20°C. Has limited stability. If older than 3 weeks make new fresh solution.

Recombinant thioredoxin reductase (TrxR)

187 μl containing 4.0 U (5.35 μM) TrxR in 50 percent glycerol, 50 mM Tris-Cl, 1 mM EDTA, pH 7.5.

Preparation procedure:

Dilute from 5.35 μM to 1.0 μM (e.g. 60 μl TrxR to 261 μl Assay Buffer).

Cap and shake the tube. Make aliquots for long term use; TrxR is not durable in low concentrations.

Store at -20°C.

Additional information:

35 U recombinant thioredoxin reductase is 1.0 mg pure enzyme (mol. mass 112,000 Da).

Human thioredoxin 1 (hTrx-1)

12 μg human recombinant hTrx-1 (lyophilized).

Preparation procedure:

Dissolve the content in 50 μl Assay Buffer to yield a final concentration of 20 μM (0.24 mg/ml, stock solution). Be careful when dissolving all material. Store at -20°C.

Additional information:

Human thioredoxin (mol. mass 12,000 Da). During storage, the protein oxidizes to a two disulfide form.

Eosin-labeled Insulin

Lyophilized Eosin-labeled Insulin.

Preparation procedure:

Dissolve the content in 1.0 ml distilled water, transfer to a larger container and add additionally 1.0 ml distilled water to a final volume of 2.0 ml. Store at -20°C. Light sensitive.

THIOREDOXIN ACTIVITY ASSAY

General information

- The final volume of the assay is 100 μl.

- The assay recording fluorescence (step 6 below) is performed at ambient temperature in the instrument.

- The assay should be carried out in triplicate but this is the choice of the user.

- Record the emission at 545 nm after excitation at 520 nm for 30 or up to 60 minutes.

- Make sure your sample is diluted to fit within your standard curve range (24-120 ng) to ensure accuracy. This can be done by testing well 2 and well 6 together with different dilutions of sample (according to well 7 & 8 in the table below) before performing the full sized assay.

Assay procedure

Comment: Always avoid bubbles when pipetting into the wells.

1. Prepare hTrx-1 to a final concentration of 12 μg/ml (1μM) by diluting hTrx-1 stock 20 times (e.g. 5 μl 0.24 mg/ml hTrx-1 to 95 μl Assay Buffer).

2. To execute the hTrx-1 assay, follow the protocol suggested in Table 1.

Table 1. Schematic example for determination of thioredoxin activity using the fluorescent substrate.

Well	1	2	3	4	5	6	7	8
Final conc. hTrx-1 in assay	-	24 ng	48 ng	72 ng	96 ng	120 ng	Sample	Sample background
Assay Buffer	65 μl	63 μl	61 μl	59 μl	57 μl	55 μl	65-x	75-x
TrxR	10 μl	10 μl	10 μl	10 μl	10 μ l	10 μl	10 μl	-
1 μM Trx	0 μl	2 μl	4 μl	6 μl	8 μl	10 μl	-	-
Sample	-	-	-	-	-	-	x	x

3. Add 5 μl β-NADPH to all wells.

4. Incubate for 30 minutes at 37°C in an incubator covering the plate with a lid.

5. After incubation is completed, add 20 μl of the fluorescent substrate to each well, and incubate for another 5 minutes.

6. Record the emission at 545 nm after 520 nm excitation for 60 minutes in a fluorescent plate reader at ambient room temperature.

7. Calculate the increasing fluorescence intensity for the time of the reaction within a linear range (Excel can be used to calculate the results).

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CALCULATION OF RESULTS

Determine the rate of reaction (Δfluorescence per minute) within the linear range for the control wells and sample by selecting two points in the linear portion of the curves as follow:
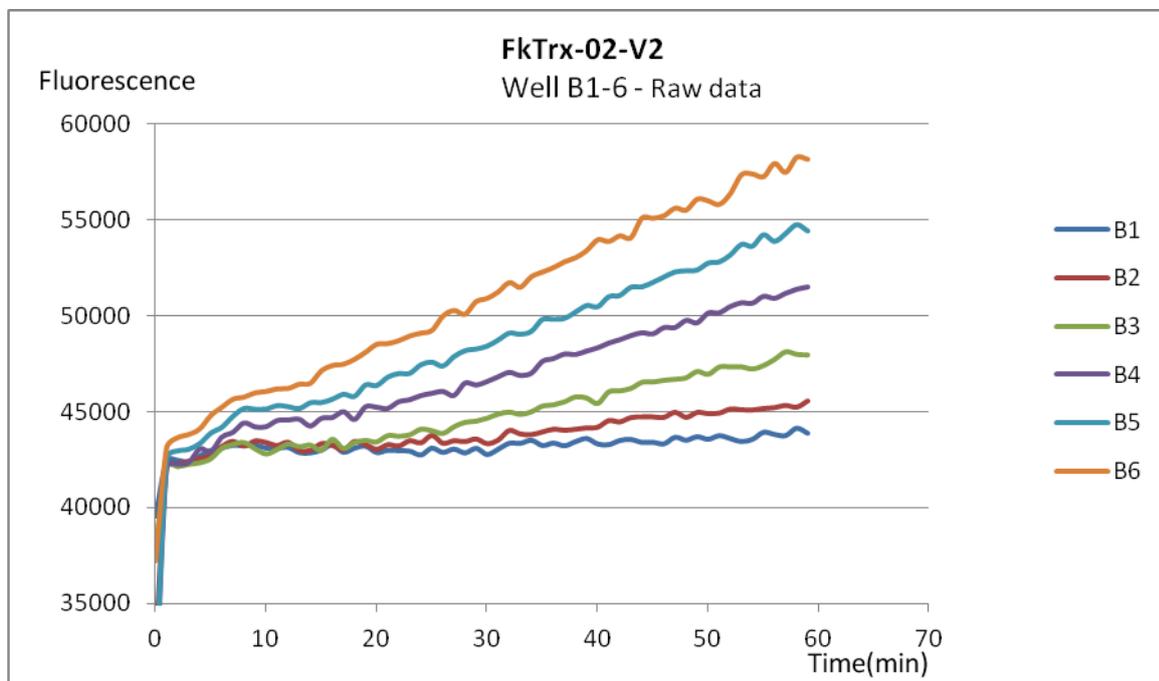
$$\frac{\Delta \text{fluorescence}}{\text{minute}} = \frac{\text{fluorescence}_{(Time\ 2)} - \text{fluorescence}_{(Time\ 1)}}{\text{Time 2}_{(min.)} - \text{Time 1}_{(min.)}}$$

To calculate the corresponded Trx activity of the sample, use the formula given by the standard curve from the experiment.

The above can be done with excel.

Example:

Make a scatter graph with connected points and use fluorescence as y-axis and time as x-axis.

FkTrx-02-V2
Well B1-6 - Raw data

The first 15 minutes are not allways linear and should therefore not be used. The recommended interval to use for your standard curve is about 15-50 (+-5) minutes.
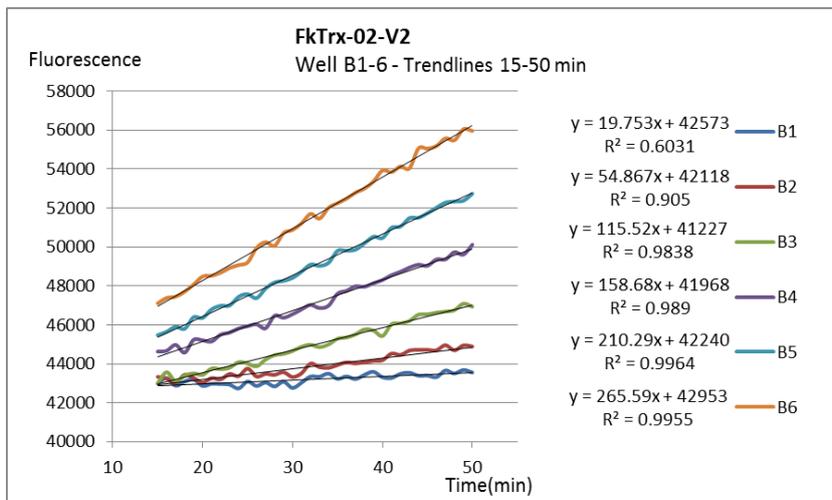
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Make a graph with the chosen interval of your standard curves. Add trend lines to be able to get the slope of each curve.

Subtract each value with the slope value of the "blank" (0ng Trx curve).
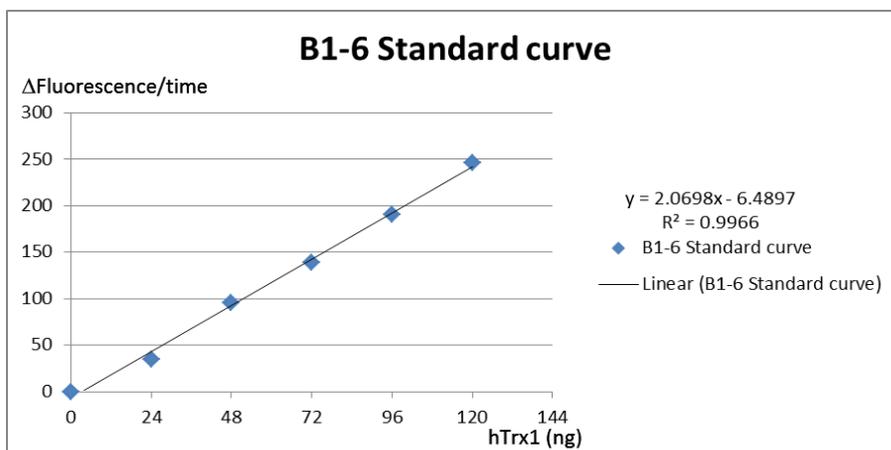
In the example assay the blank is B1.

B1 = 19.753

B1 - B1 = 0

B2 - B1 = 54.867 - 19.753 = 35.114

B3 - B1 = 115.52 - 19.753 = 95.767

…

Use the resulting values to make a scatter-graph like the one below. Use the concentration or weight of Trx as x-axis value, and the resulting value of each corresponding standard curve line subtracted by the background as y-axis value. Make a trend line between the points, and make sure to get the equation of the curve and the R^2 value.

Using the standard curve to measure the samples

Make a Fluorescence/time graph for your samples just as you did with the standard curve lines.

To measure the samples you have to be able to compare them to the standard curve, and to do that in a correct way it is important that you measure the sample's slope for the same time interval as the standard curve slopes was measured.

Make trend lines on the sample curve just like on the standard curve lines.

Do the same with the corresponding sample's background.

Subtract the background's slope from the sample's slope.

Also subtract the standard curve blank's slope from the sample.

Use the resulting slope value to calculate the concentration with the standard curve.

Example:

Standard curve equation: $y = 2.0698x - 6.4897$

Example sample equation: $y = 150x + 46000$

Example background equation: $y = 60x + 44000$

Sample slope = 150

Background slope = 60

Standard curve blank slope = 19.753

Resulting slope = Sample - Background - Blank = 150 - 60 - 19.753 = 70.247

The resulting slope is comparable to the standard curve. Use the resulting sample slope as y-value in the

standard curve equation to find x. Finding x gives concentration or ng Trx in well (depending on your standard

curve x-axis).

Standard curve equation: $y = 2.0698x - 6.4897$

$70.247 = 2.0698x - 6.4897$

$76.7367 = 2.0698x$

$x = 37.07 =$ ng hTrx1 in the sample well.

Now calculate what is in your sample stock by multiplying with your sample dilution factor.

Suggested preparation of cell lysates

i. The amount of Trx varies from different cell cultures or tissues.

ii. Collect cells (1-10 x 10^6) by centrifugation at 1,000 x g for 10 minutes.

iii. Remove supernatant (cell medium), wash with PBS and centrifuge once more at 1,000 x g for 10 minutes.

iv. Remove supernatant (PBS) and sonicate the cell pellet in 0.2 – 0.5 ml TE buffer

v. Centrifuge 10,000 x g for 20 minutes at 4 ^{o}C

vi. Remove supernatant and store it at -80°C if not assaying the same day.

vii. It is recommended to use 10-20 µg total protein from cell lysates when measuring Trx activity.

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Thioredoxin assay

The principle of the assay is the rapid reaction between reduced thioredoxin and protein disulfides. In the case of insulin, which has three disulfides, the rate of reaction is 5×10^4 M^{-1} x s^{-1}. In the assay an excess of insulin is used and the thioredoxin is kept in its oxidized form. By using NADPH and the relative excess of thioredoxin reductase, thioredoxin is reduced. Consequently, NADPH will reduce insulin disulfides via the combined reaction of the thioredoxin system yielding $NADP^+$ and reduced insulin (equation 3 in Fig 1). The thioredoxin reductase used in the kit (Rat recombinant TR-03 from IMCO) is a selenoenzyme in excess with a broad specificity reducing also Trx1 from other species like E.coli.

The assay of thioredoxin involves adding unknown samples. It is recommended that at least two different amounts are used to notice that a linear relation is achieved. Tissue levels of thioredoxin are in the range 1-20 µM calculated on the cell volume. Plasma or serum samples are generally possible to determine followed by reduction by DTT (1 mM) and desalting (9). Samples stored for a long time that have been subject to oxidative conditions, may contain over-oxidized or inactive thioredoxin. This is reactivated during the assay by the preincubation procedure.

General trouble shooting Thioredoxin is heat stable up to 70^oC and thioredoxin reductase is also stable up to about 60^oC. Both components are easily inactivated by over-oxidation. Many compounds like heavy metals, alkylating agents, SDS and aggressive chemicals used for modification of proteins will interfere and inactivate components and the sample will not be compatible for assays. This involves also electrophilic reagents and a number of drugs. The kit enables testing of drugs or interference of substances with the activity of thioredoxin or thioredoxin reductase, respectively.

Stability of reagents Generally, solutions of thioredoxin and thioredoxin reductase can be stored frozen at -20^oC. However, it may be advisable to aliquote the solutions in tubes to be thawed and used only once. Insulin, DTNB and the buffer solutions are generally stabile. NADPH will slowly oxidize and rearrange in solution also when frozen. It is therefore generally recommended to use NADPH only for one to three weeks. For this purpose, it is advisable to prepare small aliquotes of NADPH to be used up in the assays and prepare fresh NADPH from solid substance if the kit is used for a long time.

Note: The enzyme will not work with NADH. Also be sure to get the right β-NADPH and not $NADP^+$.

Abbreviations:		
	BSA (Sigma):	Bovine Serum Albumin
	β-NADPH (Sigma):	Nicotinamide adenine dinucleotide phosphate
	EDTA:	Ethylenediaminetetraacetic acid
	Trx:	Thioredoxin
	TrxR:	Thioredoxin Reductase
	Tris:	Trishydroxymethylaminomethane

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