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FkTRXR-03-Star

Kit for assay of mammalian thioredoxin reductase

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). A classical way to determine the activity of the thioredoxin reductase is by using DTNB (4,5) with NADPH as the ultimate electron donor (6). Mammalian thioredoxin reductase is a selenoprotein with a large number of substrates (7,8) in contrast to bacterial thioredoxin reductase, which is a smaller sulfur-enzyme with a very restricted specificity.

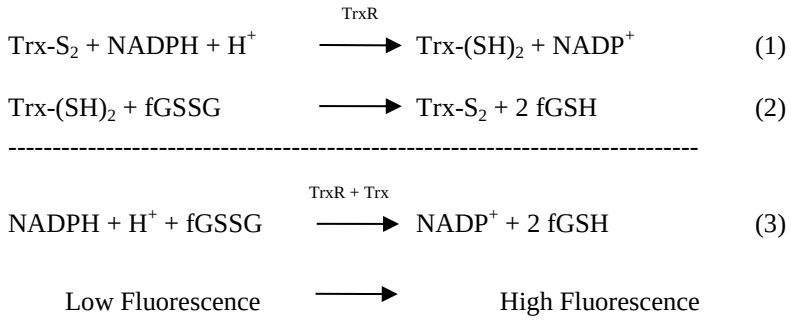

Fig 1. Scheme of reactions that form the basis of the FkTRXR-03-Star assay (9, 10).

FLUORESCENT THIOREDOXIN REDUCTASE ASSAY 96 well

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BUFFERS, SOLUTIONS AND KIT CONTENT

Required for the assay

Fluorescence micro plate reader with 520 nm excitation /and 545 nm emission like Perkin Elmer Enspire.

96 black micro titer plates (Thermo).

Supplied components

Assay Buffer

0.2 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 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.

Recombinant thioredoxin reductase (TrxR) 0.56 Units (U)

40 µl of TrxR 14 U/ml in 50 percent glycerol-50 mM Tris-Cl-1 mM EDTA, pH 7.5 (Stock solution).

Preparation procedure to make standard TrxR

Add 4 µl of TrxR to 156 µl of Assay Buffer to make 160 µl. From this take 20 µl plus 155 µl of Assay buffer to make 175 µl of 10 nM standard TrxR.

Resuspend carefully. **DO NOT VORTEX**

Additional information: Store stock solution at -20^{o}C. Make fresh standard every time.

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

Human thioredoxin 1 (hTrx-1)

0.24 mg recombinant hTrx-1 (lyophilized).

Preparation procedure:

Dissolve the content with 1.0 ml Assay buffer yielding 20µM hTrx-1. Be careful when dissolving all material. **DO NOT VORTEX**.

Store at -20°C.

Fluorescent substrate

Lyophilized fluorescent substrate

Preparation procedure:

Dissolve the content in 1.0 ml distilled water.

Store at -20°C.

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THIOREDOXIN REDUCTASE 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 it is the user's choice.
- Record the emission at 545 nm after excitation at 520 nm for 30 minutes.

Assay procedure

Comment: Always avoid bubbles when pipetting into the wells.

1. Prepare TrxR to 10 nM by diluting the TrxR stock (see page 2).
2. To execute the hTrxR assay, follow the protocol suggested in table 1.

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

Well	1	2	3	4	5	6	7	8
Final conc. TrxR in assay	Blank	0.2 nM	0.4 nM	0.6 nM	0.8 nM	1 nM	Sample	Sample background
Assay Buffer	75 µl	73 µl	71 µl	69 µl	67 µl	65 µl	75-x	85-x
20 µM Trx	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	-
10 nM TrxR	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 ^{o}C in an incubator covering the plate with a lid.
5. After incubation is completed, add 10 µl of the fluorescent substrate to each well and shake well. Using a multipipett is recommended.
6. Record the emission at 545 nm after 520 nm excitation for 30 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

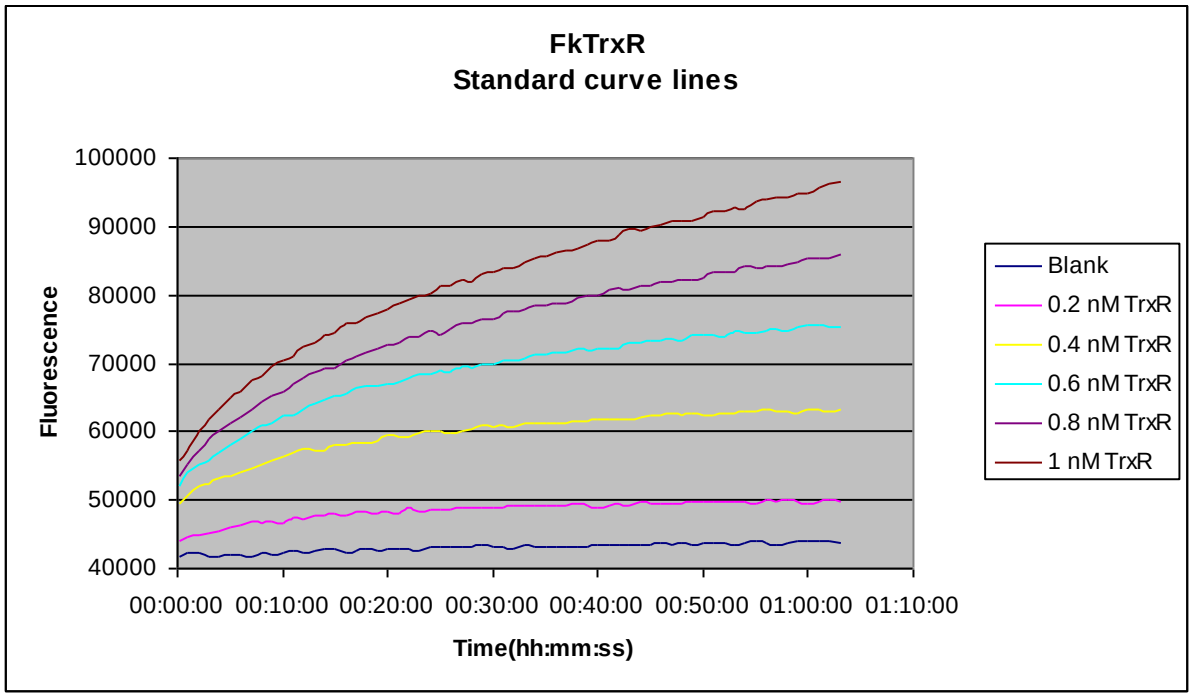
Determine the rate of reaction (Δfluorescence per minute) within the linear range for the control wells and sample by selecting two points in the linear portion of the curves as follow:

$$\frac{\Delta\text{fluorescence}}{\text{minute}} = \frac{\text{fluorescence}_{\text{(Time 2)}} - \text{fluorescence}_{\text{(Time 1)}}}{\text{Time 2}_{\text{(min.)}} - \text{Time 1}_{\text{(min.)}}}$$

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

Example:

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



The first part of the curves are the best to use. The recommended interval to use for your standard curve is about 5-15 (+-5) minutes. Use an interval the gives you a standard curve with a good R^2-value. If your do not know what an R^2-value is, keep reading and you will see how to find it.

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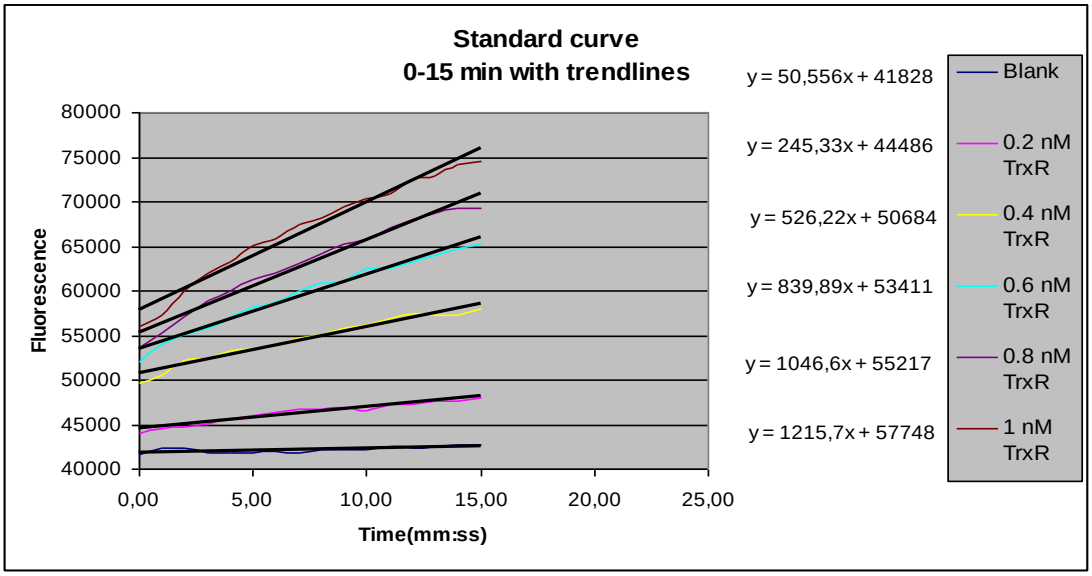
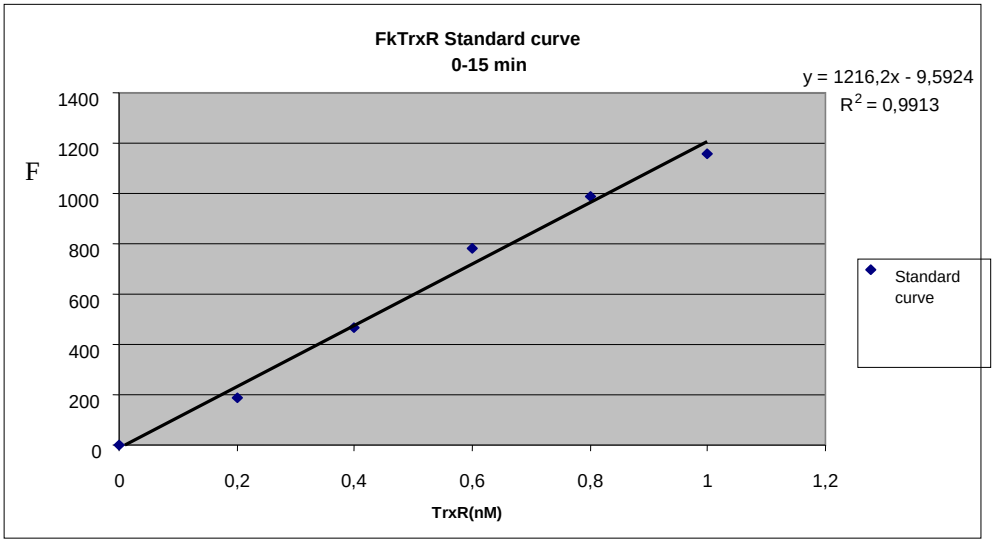


Make a graph with the chosen interval of your standard curves. Then add trendlines to be able to get the slope of each curve.

Subtract each value (even the blank) with the slope value of the blank curve. In this case, this would give us:

0; 188.774; 469.664; 783.334; 990.044; 1159.144

Use the resulting values to make a point-graph like the one below. Use the concentration or weight of TrxR in the standard lines as x-axis and the resulting value of each corresponding standard curve line, subtracted by the blank, as y-axis value. Make a trendline between the points, and make sure to get the equation of the curve and the R^2 value. The higher R^2-value, the more precise you will be able to determine your sample's concentration.



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Using the standard curve to measure the samples

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

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

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

Do the same with the corresponding sample's background.

Subtract the background slope (k-value) from the sample slope(k-value).

Use the resulting slope value (k-value) to calculate the concentration with the standard curve.

Example:

Standard curve equation: y= 1216.2x -9.5924

Example sample equation: y=900+50000

Example background equation: y=100+40000

Sample slope =900

Background slope=100

Resulting slope=800=y value on standard curve point-graph

Standard curve= y = 1216.2x+ 33,494 = 800

Finding x gives concentration or mass of TrxR in well(depending on your standard curve x-axis):

1216.2x+ 33,494 = 800

1216.2x = 766.506

x=0.63nM TrxR

Suggested preparation of cell lysates

i. The amount of TrxR varies from different cell cultures.
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 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^{o}C if not assaying the same day.
vii. It is recommended to use 10-20 µg total protein from cell lysates when measuring TrxR activity.

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Thioredoxin reductase assay

The principle of the assay is the rapid reaction between reduced thioredoxin and disulfides.The preincubation serves to activate thioredoxin by reduction. By using NADPH and the relative excess of disulfide thioredoxin is oxidized. It is then reduced by thioredoxin reductase which is rate-limiting. Consequently, NADPH will reduce fluorescent glutathione via the combined reaction of the thioredoxin system yielding $NADP^+$ and fluoresent GSH with high fluorescence (see Reactions 1-3).

The assay of thioredoxin reductase 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 reductase are in the range 0.1-1.0 µM calculated on the cell volume.

General trouble shooting

Thioredoxin is heat stable up to 70°C and thioredoxin reductase is also stable up to between 60 and 70°C. 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°C. 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, it is therefore generally recommended to be used only for one to three weeks. For this purpose, it is advisable to prepare a small aliquotes of NADPH to be used up in the assays and prepare fresh NADPH. Even when frozen NADPH solutions decompose.

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 phospate
	EDTA:	Ethylenediaminetetraacetic acid
	HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
	Trx:	Thioredoxin
	TrxR:	Thioredoxin Reductase
	Tris:	Trishydroxymethylaminomethane
	fGSSG:	Fluoresent glutathione disulfide
	fGSH:	Fluorescent glutathione

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