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Kit for assays of mammalian Trx and TrxR

The thioredoxin system is the major protein disulfide reductase in cells and comprises thioredoxin, thioredoxin reductase and NADPH (1). Thioredoxin systems are ubiquitous (1, 2) and are critical for cell viability (2, 3), proliferation (2, 4), activation of transcription factors (2, 5) and control of intracellular redox states (6, 7). The activity of the thioredoxin system have been reported to be disrupted in various physiological disorders such as HIV infection (8, 9), Alzheimer disease (10) and diverse types of cancer (11-15) and is therefore useful as a biological marker for several diseases. The classical way to determine the activity of the thioredoxin system is by insulin assays (6, 7). This method is based on the reduction of insulin disulfides by reduced thioredoxin with thioredoxin reductase 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 (17). There are currently two approaches to record the activity of the thioredoxin system, either by measuring the consumption of NADPH (A_{340}) or by measuring the formation of thiols (16). For this purpose [5,5'-dithiobis-(2-nitrobenzoic acid] DTNB (for abbreviations see page 5) is used to determine the number of free thiols from reduced insulin, where reduction of one mol (DTNB) leads to the formation of two mol of thionitrobenzoate (TNB; molar extinction coefficient 13600 M^{-1} cm^{-1} at 412 nm) a yellow product measured at A_{412} nm.

$$\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{\text{TrxR}} \text{Trx-(SH)}_2 + \text{NADP}^+ \quad (1)$$

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

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

$$\text{Insulin-(SH)}_2 + \text{DTNB} \longrightarrow \text{Insulin-S}_2 + 2\text{TNB} \quad (4)$$

Fig 1. Scheme of reactions that form the basis of the insulin assay.

Thioredoxin endpoint 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 x 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 reaction is broken by using 8 M guanidine hydrochloride and DTNB, which will denature and inactive thioredoxin and thioredoxin reductase and derivative all SH-groups to yield TNB. The assay is based on the net increase of free sulfhydryl groups in the reaction. In tissue or cell extracts there may be sulfhydryl containing proteins, GSH or other sources of SH-groups, which have to be subtracted by running blank determinations.

The assay is sensitive and linear in a range as specified in this description. Through extensive development it has been possible to obtain a reproducible assay using a large excess of EDTA (20 mM) and by diluting the enzymes using bovine serum albumin. This prevents the absorption of thioredoxin and thioredoxin reductase to plastic surfaces used in the 96-well plates as well as in pipette tips.

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The human thioredoxin is produced recombinantly in *E. coli* and has been lyophilized from Tris-EDTA. The recombinant thioredoxin reductase from rat is produced and shipped as an 85 percent ammonium sulfate suspension, where it is stable. The reagents in the assay involving insulin solutions, NADPH and buffer solutions will have to be prepared by the user.

Thioredoxin assay

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 not possible to determine unless concentrated. 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, but will result in a lag phase. Consequently, the content of thioredoxin may be underestimated. A solution to this is to incubate the sample with dithiothreitol (0.5-1 mM). Observe that it is required to subtract a blank for the added DTT.

Thioredoxin reductase assay

The principle of thioredoxin reductase assay is based on using a relative excess of thioredoxin (50 µM). The K_m-value of thioredoxin for thioredoxin reductase is around 2 µM. The turnover of the enzyme (all calculations on the enzyme in the kit is based on pure enzyme) is 3000 min^{-1}.

General trouble shooting

Thioredoxin is heat stable up to 70^{o}C and thioredoxin reductase is also stable up to between 60 and 70^{o}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^{o}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.

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

Buffers and solutions:

Required for the assays (not provided with kit):

- Mixture M: 210 mM HEPES pH 7.6
  790 µM Insulin (4.55 mg/ml)
  20 mM EDTA
  (Can be frozen after use for further experiments)

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- Stop A: 8 M GuHCl in 0.2 M Tris-Cl pH 8.0 (stable at room temperature)

- Stop B: 10 mM DTNB in 99.5% ethanol (keep tightly sealed at -20°C)

- TE-buffer (pH 7.6): 50 mM Tris-Cl
20 mM EDTA

- β-NADPH (solution N): 50 mM (40 mg/ml) (stored at -20°C)

To perform assay, a micro plate reader and 96-well microtiter plates or ELISA strips are required

Provided with kit:
TrxR ampoule

- Standard (Std) TrxR: 5 units in 250 µl (0.15 mg of active enzyme) (keep at -20°C)

Content: 0.15 mg TrxR (5 units) in 250 µl 50 percent glycerol and TE buffer.

Preparation procedure:
Add 1.75 ml TE buffer to yield a final concentration of 0.65 µM (75 µg/ml).

Trx ampoule

- Standard (Std) Trx: 0.6 mg/ml (50 µM) (keep at -20°C)

Content: 1.2 mg human recombinant Trx1 (lyophilized)

Preparation procedure:
Dissolve the ampoule content in 2000 µl TE to yield a final concentration of 50 µM (0.6 mg/ml).

Preparation of 1.6 mM (10 mg/ml) insulin:
Suspend 10 mg insulin (pig , human or bovine) in 0.5 ml of 50 mM Tris-Cl, pH 7.5. Adjust pH to 2 to 3 with small additions of 1 M HCl to dissolve the protein completely, and slowly titrate back to the original pH of the buffer with 1 M NaOH. Finally adjust the volume with distilled water to 1 ml. This clear stock solution of insulin (10 mg/ml) can be kept at -20^{o}C.

Comment: When titrating back to the original pH of the buffer the solution will become turbid. Proceed until the pH of the buffer is reached and the solution turns transparent.

Thioredoxin assay:

1. Add 20 µl solution N (40 mg/ml β-NADPH) to 480 µl mixture M → reaction mixture "M+N" (for 24 samples)
2. Prepare reaction mixtures and standard curves (suggested in Table 1; all reagents should be kept on ice):

- For the standard curve (Table 1) dilute the Std hTrx 200-fold to yield a final concentration of 3 ng/µl using TE buffer containing 1 mg/ml BSA (i.e. 1 µl Std Trx + 199 µl buffer).
- The added protein amount should contain 5-14 µg total protein amount in a maximum volume of 20 µl (x µl; Table 1) when analyzing complex samples such as cell or tissue cell free extracts. If the sample needs to be diluted, use TE buffer (20-x µl).

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- For correction of endogenous thiols, a well with out TrxR is required (blank; 30-x µl).

3. Carefully add TrxR and TE buffer or protein sample in each well and add 20 µl of reaction mixture "M+N" to each well. Mix and incubate immediately for 30 min at 37°C.
- Blank and samples have to reach a final volume of 50 µl, respectively, in each well

Table 1: Schematic example of thioredoxin assay

	Background	Standard curve					Blank / Sample	
TrxR (0.65 µM)	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl		10 µl
TE	20 µl	19 µl	18 µl	17 µl	16 µl	15 µl	30-x µl	20-x
hTrx (3 ng/µl)	-	1 µl (3.0ng)	2 µl (6ng)	3 µl (9ng)	4 µl (12ng)	5 µl (15ng)	-	-
Sample	-	-	-	-	-	-	x µl	x µl
M + N	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl
Total Volume	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**

4. During incubation, prepare Stop A+B by mixing Stop A with Stop B in a ratio of 9:1 (i.e 9 ml Stop A and 1 ml Stop B). At the end of incubation, add 200 µl Stop A+B to each well. This will denature all proteins, stop enzymatic reactions and derivatize all thiols with DTNB, releasing free TNB, which is yellow and absorbs light at 412 nm.

5. When all reactions have been terminated, read the absorbance at 412 nm of the standard curve and the samples using a micro plate reader.

6. Subtract the absorbance of the background from the absorbance of the standard curve and use the corrected absorbance values of to plot a standard curve, using the appropriate amounts of Trx. For the samples, subtract the corrected absorbance of blank from sample for each unknown sample and use that value for determination of the amount of Trx in the sample, utilizing the corresponding standard curve. The absorbance of blank is subtracted to avoid interference from endogenous thiol content in the samples.

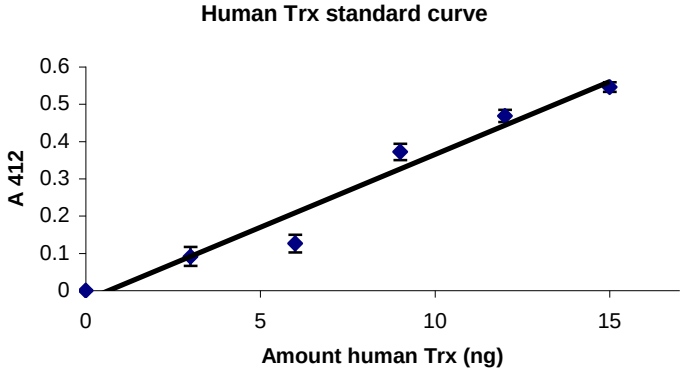

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Fig 1. Standard curve for human thioredoxin

Thioredoxin Reductase assay:

1. Add 20 µl solution N (40 mg/ml β-NADPH) to 480 µl mixture M → reaction mixture "M+N" (for 24 samples)
2. Prepare reaction mixtures and standard curves (suggested in Table 2; all reagents should be kept on ice):

- For the standard curve (Table 2) dilute the TrxR 40 times to yield a final concentration of 1.87 ng/µl using TE buffer containing 1 mg/ml BSA (i.e. 1 µl TrxR + 39 µl buffer).
- The added protein amount should contain 5-14 µg total protein amount in a maximum volume of 20 µl (y µl; table 2) when analyzing complex samples such as cell or tissue cell free extracts. If the sample needs to be diluted, use TE buffer (20-y)
- For correction of endogenous thiols, a well with out Trx is required (blank; 30-y µl).

3. Carefully add Trx and TE buffer or protein sample in each well and add 20 µl of reaction mixture "M+N" to each well. Mix and incubate immediately for 30 minutes at 37°C.

- Blank and sample has to reach a final volume of 50 µl respectively in each well.

Table 2: Schematic example of thioredoxin Reductase assay

	Background	Standard curve					Blank / Sample	
Trx (50 µM)	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl		10 µl
TE	20 µl	19 µl	18 µl	17 µl	16 µl	15 µl	30-y µl	20-y
TrxR (1.87 ng/µl)	-	1 µl (1.87ng)	2 µl (3.75ng)	3 µl (5.62ng)	4 µl (7.5ng)	5 µl (9.37ng)	-	-
Sample	-	-	-	-	-	-	y µl	y µl
M + N	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl
Total Volume	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**

4. During incubation, prepare Stop A+B by mixing Stop A with Stop B in a ratio of 9:1 (i.e 9 ml Stop A and 1 ml Stop B). At the end of incubation, add 200 µl Stop A+B to each well. This will denature all proteins, stop enzymatic reactions and derivatize all thiols with DTNB, releasing free TNB, which is yellow and absorbs light at 412 nm.

5. When all reactions have been terminated, read the absorbance at 412 nm of the standard curve and the samples using micro plate reader.

6. Subtract the absorbance of the background from the absorbance of the standard curve and use the corrected absorbance values of to plot a standard curve, using the appropriate amounts of TrxR. For the samples, subtract the corrected absorbance of blank from sample for each unknown sample and use that value for determination of the amount of TrxR in the sample, utilizing the corresponding standard curve. The absorbance of blank is subtracted to avoid interference from endogenous thiol content in the samples.

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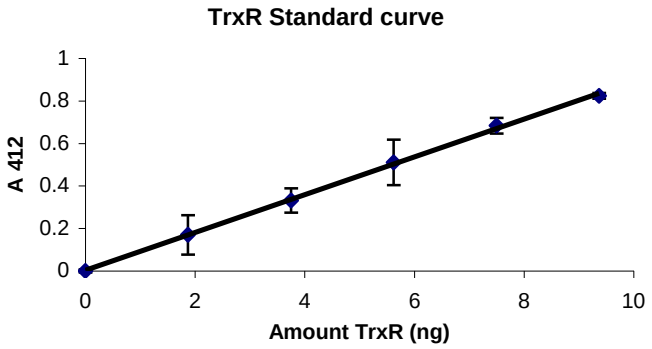

Fig 2. Standard curve for thioredoxin reductase

Abbreviations:

BSA (Sigma):	Bovine Serum Albumin
β-NADPH (Sigma):	Nicotinamide adenine dinucleotide phosphate
DTNB:	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA:	Ethylenediaminetetraacetic acid
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
GuHCl:	Guanidine hydrochloride
TNB:	Thionitrobenzoate
Trx:	Thioredoxin
TrxR:	Thioredoxin Reductase
Tris:	Trishydroxymethylaminomethane

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