Technical Bulletin

Cytochrome c Reductase (NADPH) Assay Kit

Catalogue number CY0100

Product Description

This kit is designed to measure the NADPH cytochrome c reductase activity in biological samples including cell and tissue extracts, and in purified microsomes (endoplasmic reticulum, ER).

Eukaryotic NADPH-Cytochrome c reductase (NADPH cytochrome P450 reductase, EC 1.6.2.4) is a flavoprotein localized to the endoplasmic reticulum. It transfers electrons from NADPH to several oxygenases, the most important of which is the cytochrome P450 family of enzymes, responsible for xenobiotic metabolism. NADPH-cytochrome c reductase is widely used as an endoplasmic reticulum marker and as a biomarker of ecological pollution and dietary lipid uptake.

This kit is based on a colorimetric assay that measures the reduction of cytochrome c by NADPH-Cytochrome c reductase in the presence of NADPH. The reduction of cytochrome c results in the formation of distinct bands in the absorption spectrum and the increase in absorbance at 550 nm is measured with time.

This kit has been tested on samples prepared from mammalian tissues such as liver, kidney, brain, spleen, and heart muscle from several species, as well as on lysates from cell lines such as HeLa, HepG2, and Jurkat. In addition, the kit has been tested on samples prepared from yeasts, *P. pastoris* and *S. cerevisiae*.

The kit is suitable for:

- Detection of ER during isolation and subcellular fractionation by density gradient separation (Endoplasmic Reticulum Isolation Kit, Catalog Number ER0100)
- Measuring NADPH-cytochrome c reductase levels in biological samples after exposure to drugs or other xenobiotics.

Components

The kit is sufficient for 100 assays.

- Assay Buffer 100 ml
  (Catalog Number A8477) 300 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA
- Enzyme Dilution Buffer 25 ml
  (Catalog Number E0155) 300 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 0.5 mg/ml bovine serum albumin
- Cytochrome c 50 mg
- NADPH 25 mg
  (Catalog Number MUS84)
- Cytochrome c Reductase (NADPH) Positive Control, from rabbit liver 50 µl
  (Catalog Number C9363)
- Cytochrome c Oxidase Inhibitor Solution 1 ml
  50 mM potassium cyanide (KCN) in water
  (Catalog Number C9238)

Equipment Required but Not Provided

- 1 ml cuvette
- Spectrophotometer
- Analytical balance

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

This kit ships on dry ice and storage at –20 °C is recommended. When stored unopened, the components are stable for 24 months.

Preparation Instructions

Reagent Preparation

These instructions are for the preparation of working solutions sufficient for 20 assays. Use ultrapure (17 MΩ·cm) water in all cases.

1. Working Solution – Prepare a Working Solution (0.45 mg/ml, 36 µM) by adding 9 mg of Cytochrome c to 20 ml of the Assay Buffer (Catalog Number A8477). Bring the Working Solution to 25 °C prior to use. This solution is stable for several hours at this temperature and may be kept overnight at 4 °C and warmed to 25 °C prior to use the next day.
2. NADPH Solution – Prepare a stock solution (40 mg/ml) by dissolving the contents (25 mg) of the bottle of NADPH (Catalog Number MUS84) in 0.625 ml of water. The stock solution may be kept at –20 °C for up to 6 months. It is recommended to aliquot the stock solution before the initial freezing step. The stock solution is further diluted (22 µl of stock solution per ml of water) to a working NADPH Solution (0.85 mg/ml) just before use. This working NADPH Solution may be kept at 25 °C for up to 2 hours, but should be discarded after this time.
3. Positive Control - Dilute an aliquot of the Cytochrome c Reductase (NADPH) (Catalog Number C9363) 10-fold with the Enzyme Dilution Buffer just before assaying. Each set of reactions requires a total of 75 µl of the diluted Positive Control. The diluted Positive Control may be stored at 4 °C for up to 5 days.
4. Sample Preparation:
   Detergents used to extract biological samples may have an effect on the activity of cytochrome c reductase. CHAPS, sodium cholate, sodium deoxycholate, and TRITON® X-100 at concentrations up to 0.05% in the final reaction mixture have been tested with this procedure and were found to be compatible with the assay procedure. When a detergent is used to lyse cells or to extract tissue, it may cause the release of cytochrome c reductase from the ER membrane into the soluble fraction. Centrifugation at 100,000 x g will result in virtually all the activity in the supernatant and very little in the pellet.

   Animal Tissues – Microsomes may be prepared using the Endoplasmic Reticulum Isolation Kit (Catalog Number ER0100). Alternatively, homogenize the tissue sample in an isotonic buffer, pH 7.5. Centrifuge sequentially at 1000 x g and 12,000 x g to obtain the post-nuclear and post-mitochondrial supernatants, respectively. The microsomal pellet is obtained by centrifuging the 12,000 x g supernatant for 1 hour at 100,000 x g. The amount of sample required depends on the expected level of the enzyme in the sample. See Appendix for recommended amounts of extracts for a 1-minute enzyme assay.

   Cell Lines - For cell culture samples mechanical breakage can be performed in an isotonic buffer by passage through a 27-gauge needle or by homogenization in a Dounce homogenizer. Alternatively, the cells may be swollen in a hypotonic buffer, adjusted to isotonic conditions, and then broken with the aid of a Dounce homogenizer. Centrifuge in a manner similar to the animal tissue preparation.

   Yeast - Cells can be lysed with the aid of Lyticase (Catalog Number L8658) at a concentration of 625 units per gram of cells. After incubation at 37 °C, the spheroplasts should be washed, suspended in an isotonic buffer, and homogenized with a PTFE and glass homogenizer. The time of incubation required for spheroplast formation should be determined for each yeast strain (15-45 minutes). Centrifuge in a manner similar to the animal tissue preparation.

Procedure

This assay measures the reduction of cytochrome c by NADPH-cytochrome c reductase in the presence of NADPH. The absorption spectrum of cytochrome c changes with its oxidation/reduction state. Upon reduction a sharp absorption peak is observed at 550 nm. The reduction of cytochrome c is monitored by the increase of cytochrome c absorbance at 550 nm. **Note that the monitored wavelength is critical and the deviation must be 2 nm or less. A deviation of 10 nm will result in no signal at all.** The assay buffer provided has been determined to be optimal for the enzyme activity.

The activity is measured by a spectrophotometer with a kinetic program and is linear for 3-5 minutes when 1-4 milliunits of enzyme are added per assay. The linearity range is 1-6 milliunits for a 1-minute enzyme assay.

In crude extracts of tissues or cell lines (1000 x g supernatant), excessive shearing forces during preparation can expose the inner mitochondrial membrane that contains cytochrome c oxidase. This

enzyme catalyzes the reverse reaction (oxidation of cytochrome c) under the assay conditions and will cause inaccurate determination of reductase activity. This reaction can be prevented by addition of 20 µl of the Cytochrome c Oxidase Inhibitor Solution (50 mM potassium cyanide solution) to the reaction mixture. This results in a final potassium cyanide concentration of 1 mM.

Soft tissues such as liver, spleen, and brain show no increase in reductase activity when assayed in the presence of KCN indicating no significant levels of cytochrome c oxidase present. Hard tissues show a large increase in reductase activity upon addition of KCN (heart muscle, 3.6-fold and kidney, 1.7-fold). Yeast cells lysates prepared with lyticase contain no significant amounts of cytochrome c oxidase and it is not necessary to add the inhibitor solution. Purified microsomes (100,000 x g pellet) do not require the addition of KCN, since they are normally free of any mitochondrial contamination.

Cytochrome c Reductase Assay

Keep the enzyme preparations (samples and control) on ice. Warm the Working Solution and the working NADPH Solution to 25 °C before use. Run each test separately.

Note: Reagent volumes for blank, control, and test samples are summarized in Table 1.

1. Set the spectrophotometer to 550 nm and run the kinetic program at 25 °C:
   Initial delay = 5 seconds
   Interval= 10 seconds
   Readings = 7
   For very dilute samples increase the reading time up to 5 minutes.
2. Place 950 µl of Working Solution (at 25 °C) in a 1 ml cuvette and add up to 50 µl of sample. If the sample size is less than 50 µl, use Enzyme Dilution Buffer to bring the volume to 1 ml.
3. For samples with expected interference from cytochrome c oxidase activity, add 20 µl of the Cytochrome c Oxidase Inhibitor Solution.
4. For the positive control reactions, add 25 or 50 µl of the prepared Positive Control.
5. Mix by inversion and start the reaction by addition of 100 µl of NADPH Solution. Mix by inversion again.
6. For a blank reaction measure the value given by the reagents alone without enzyme present.
7. Calculate the activity of the samples assayed.

Table 1.
Reaction Scheme

Test	Working Solution	Enzyme Dilution Buffer	Sample or Positive Control	NADPH Solution 0.85 mg/ml	Inhibitor solution
Blank	950 µl	50 µl	___	100 µl	___
unknown sample	950 µl	50-X µl	X µl	100 µl	___
unknown sample with interference	950 µl	30-X µl	X µl	100 µl	20 µl
Positive control (10-fold dilution)	950 µl	25 µl	25 µl	100 µl	___
Positive control (10-fold dilution)	950 µl	___	50 µl	100 µl	___

Results

Calculation

Unit definition: One unit will reduce 1.0 µmole of oxidized cytochrome c in the presence of 100 µM NADPH per minute at pH 7.8 at 25 °C.

$$\frac{Units}{ml} = (\Delta A_{550}/\min \times dil \times 1.1) \div (21.1 \times Enzvol)$$

$$\frac{\Delta A_{550}}{min} = \Delta A_{sample} - \Delta A_{blank}$$

dil = the dilution factor of the original enzyme sample
Enzvol = volume of the enzyme sample (ml)

21.1 = extinction coefficient (ε^{mM}) for reduced cytochrome c

1.1 = reaction volume (ml)

In the event the reaction time is longer than 1 minute, divide the ΔA_{550} by the reaction time to obtain ΔA_{550}/min.

References

1. IUBMB enzyme Nomenclature
2. Shen, A.L., and Kasper, C.B., Protein and gene structure and regulation of NADPH-Cytochrome P450 oxidoreductase, in Handbook of Experimental Pharmacology, Vol. 105, Schenkman, J.B., and Greim, H., eds., Springer, (New York, NY: 1993) pp. 35-59.
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4. Vidal. M-L., et al., Seasonal variations of pollution biomarkers in two populations of Corbicula fluminea (Muller), Comp. Biochem. Physiol. Part C, 131, 133-151 (2002).
5. Hochgraf, E., et al., Dietary oxidized linoleic acid modifies lipid composition of rat liver microsomes and increases their fluidity. J. Nutr., 127, 681-686 (1997).
6. Vermillion, J.L., and Coon, M.J., Highly purified detergent solubilized NADPH-Cytochrome P-450 reductase from Phenobarbital-induced rat liver microsomes. Biochem. Biophys. Res. Commun., 60, 1315-1322 (1974).
7. Buege, J.A., and Aust, S.D., Comparative studies of rat liver and lung NADPH-Cytochrome c reductase. Biochim. Biophys. Acta, 385, 371-379 (1975).

Appendix Table 2.
Levels of cytochrome c reductase in various cell lines.

Cell line	Fraction	µl/assay	units/ml of extract	units per 10^6 cells
HeLa	1,000 × *g* supernatant	50	9.8×10^{-3}	1.37×10^{-5}
	12,000 × *g* supernatant	50	5.2×10^{-3}	4.60×10^{-6}
HepG2	1,000 × *g* supernatant	50	1.02×10^{-1}	4.86×10^{-3}
	12,000 × *g* supernatant	50	2.99×10^{-2}	1.23×10^{-3}
Jurkat	1,000 × *g* supernatant	25	1.67×10^{-2}	6.68×10^{-5}
	12,000 × *g* supernatant	25	1.7×10^{-2}	6.12×10^{-5}
	12,000 × *g* pellet	10	4.43×10^{-2}	1.77×10^{-5}
	100,000 × *g* pellet	10	3.65×10^{-2}	1.32×10^{-4}

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