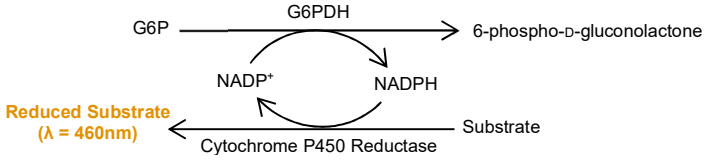
Cytochrome P450 Reductase (CPR) Activity Kit (Colorimetric) (#BN00924)

(Catalog # BN00924; 100 Reactions; Store at -20°C)

I. Introduction:

NADPH-cytochrome P450 reductase (CPR, EC 1.6.2.4) is a ~78 kDa membrane-bound flavoenzyme that catalyzes the transfer of electrons from NADPH to members of the cytochrome P450 monooxidase (CYP) enzyme family in the endoplasmic reticulum. CPR contains two tightly bound flavin cofactors, FAD and FMN, which participate in the sequential transfer of electrons from NADPH→FAD→FMN→CYP, oxidizing NADPH to $NADP^+$ and reducing the CYP heme moiety to the substrate- and oxygen-binding ferrous state. As CPR is required for the function of all CYP isozymes, it plays a critical role in the metabolism of drugs, organic pollutants and other xenobiotic compounds, in addition to its role in biosynthesis of certain vitamins and steroid hormones. Assay Genie's cytochrome P450 reductase activity assay kit couples oxidation of NADPH by CPR to reduction of a nearly colorless probe into a brightly colored product with an absorbance peak at 460 nm, with the rate of color generation being directly proportional to CPR activity. The NADPH utilized by CPR is generated *in situ* from β-$NADP^+$ via oxidation of glucose-6-phosphate (G6P) to 6-phospho-D-glucono-1,5-lactone by glucose-6-phosphatase dehydrogenase (G6PDH). The kit can be used to determine CPR activity in a variety of samples, with a detection limit of ~0.2 mU of CPR activity per reaction. For assessment of CPR activity in crude biological samples that may have extraneous reductases capable of reducing the substrate, an inhibitor of NADPH-dependent flavoproteins is included. In this case, the specific CPR activity may be calculated by running parallel reactions in the presence and absence of inhibitor and subtracting any residual activity detected with the inhibitor present. The kit contains sufficient reagents for performing 100 reactions in a 96-well plate format.



II. Applications:

- Measurement of cytochrome P450 reductase activity in various tissues and eukaryotic cells in a 96-well plate format
- Verification of CPR enzymatic activity in purified microsomes or endoplasmic reticulum fractions

III. Sample Type:

- Purified recombinant or native CPR protein
- Human and animal liver microsomes
- Lysates of tissues and cultured eukaryotic cells

IV. Kit Contents:

Components	BN00924	Cap Code	Part Number
CPR Assay Buffer	50 ml	NM	BN00924-1
G6P Standard	1 vial	Blue	BN00924-2
Inhibitor (Diphenyleneiodonium Chloride, 10 mM)	100 µl	Clear	BN00924-3
NADPH Substrate Mix	1 vial	Amber	BN00924-4
G6P Standard Developer	1 vial	Red	BN00924-5
Human CPR Positive Control	1 vial	Green	BN00924-6

V. User Supplied Reagents and Equipment:

- Multiwell plate-based spectrophotometer capable of reading absorbance in kinetic mode
- Precision multi-channel pipette with disposable tips
- Clear 96-well plate with flat bottom

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Allow the CPR Assay Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure. Use the kit within 2 months of reagent reconstitution.

- **G6P Standard:** Reconstitute with 300 µl dH_2O to generate a 100 mM (100 nmole/µl) G6P stock solution. Keep on ice while in use. Store at -20°C.
- **Inhibitor:** The Inhibitor is provided as a 10 mM solution in DMSO. Warm the 10 mM inhibitor solution to room temperature to melt DMSO and vortex to ensure inhibitor is completely dissolved. Mix 100 µl of the 10 mM solution with 900 µl of CPR Assay Buffer to obtain final 1 mM inhibitor working solution. Store working solution at -20°C.
- **NADPH Substrate Mix:** Reconstitute with 600 µl CPR Assay Buffer, aliquot if desired and store at -20°C. Avoid repeated freeze/thaw cycles. Keep NADPH Substrate Mix on ice while in use.
- **G6P Standard Developer:** Dissolve in 600 µl dH_2O and pipette up and down to mix thoroughly. Store at -20°C.
- **Human CPR Positive Control:** Reconstitute with 50 µl CPR Assay Buffer, pipette up and down until fully resuspended (do not vortex). Keep on ice while in use. For best results, we recommend using the reconstituted CPR positive control within one day; however, it may be aliquoted and stored at -80°C if desired. Avoid repeated freeze/thaw cycles.

VII. Cytochrome P450 Reductase Activity Assay Protocol:

1. Standard Curve Preparation:

a. Prepare a 1 mM (1 nmole/µl) G6P standard solution by adding 10 µl of the 100 mM G6P stock solution to 990 µl of CPR Assay Buffer and vortexing. Add 0, 2, 4, 6, 8 and 10 µl of the G6P standard solution into a series of wells on a 96-well plate. Adjust the volume to 90 µl/well with CPR Assay Buffer to generate 0, 2, 4, 6, 8 and 10 nmole/well G6P standards.

b. To each of the standard wells, add 5 µl of NADPH Substrate Mix and 5 µl of G6P Standard Developer to make the final volume 100 µl/well. Mix the well contents using a multichannel pipette and incubate the G6P standards for at least 30 min. at room temperature, protected from light.

c. Measure absorbance at 460 nm for all of G6P standard wells and plot the G6P standard curve by subtracting the OD_{460} value of the 0 nmole/well G6P standard from all the other standard readings.

Note:

- G6P standard solution 1 mM (1 nmole/µl) can be stored at -20°C for later use. It is stable for two months at -20°C or for ~3 freeze/thaw cycles. Keep the G6P standard solution on ice while in use.

2. Sample Preparation: Microsomes may be prepared from soft tissues or cultured eukaryotic cells using the Microsome Isolation Kit. Alternatively, a crude microsomal preparation can be used: start with ~50 mg tissue or ~5 x 10^6 pelleted, pre-washed cells and homogenize in 500 µl ice-cold Assay Buffer with a Dounce homogenizer on ice. Incubate the homogenate on ice for 5 min. and then centrifuge at 1500 x *g* for 5 min. in a refrigerated centrifuge at 4°C. Transfer the supernatant to a new pre-chilled microfuge tube and centrifuge at 12,000 x *g* for 5 min. at 4°C. Collect the resultant clarified supernatant for the assay and store on ice until use (cell and tissue lysates can also be stored at -80°C in aliquots for future experiments).

Notes:

- We recommend adding a protease inhibitor cocktail to the buffer prior to tissue or cell homogenization.
- Any commercial lysis buffer containing a mild non-ionic detergent can also be used for lysis of tissues and cultured cells. In our experience, lysis buffers with similar composition to the CPR Assay Buffer containing 0.1% Triton X-100 (final concentration ≤0.05%) do not interfere with the assay.
- To quantify specific CPR activity in terms of sample protein content, save a small aliquot of the sample and quantify the protein concentration using the Bradford reagent or an equivalent protein assay.

3. Reaction Preparation:

a. Prepare reaction wells containing test samples, inhibitor control samples and (if desired) background and positive controls:

	Sample	+Inhibitor	Positive Control	Background Control
Sample (Tissue or Cell Lysate)	5–40 µl	5–40 µl	--	5–40 µl
Inhibitor (1 mM in 10% DMSO)	--	10 µl	--	--
Recombinant Human CPR	--	--	5 µl	--
CPR Assay Buffer	to 60 µl	to 60 µl	to 60 µl	to 70 µl

b. For each well, prepare a total of 30 µl reaction mixture containing 5 µl of NADPH Substrate Mix and 25 µl of CPR Assay Buffer. Add 30 µl of the assay reaction mixture to each well and mix using a multi-channel pipette. Incubate for 5 min. at room temperature to allow the Inhibitor to bind targets.

4. Measurement: Make a 20 mM G6P reaction solution by diluting the 100 mM G6P stock solution with CPR Assay Buffer at a 1:5 ratio (for example, mix 100 µl of the 100 mM G6P stock with 400 µl CPR Assay Buffer to yield 500 µl of 20 mM G6P reaction solution). Add 10 µl of the 20 mM G6P solution to each well containing sample, inhibitor control or positive control (do not add G6P to the background control) and immediately measure absorbance (OD at 460nm) in kinetic mode at 25°C for 25-30 min.

Notes:

- Since the reaction starts immediately after the addition of G6P, it is essential to preconfigure the spectrophotometer settings and use a multichannel or repeating pipette to minimize lag time among wells.
- For maximum temporal resolution, we recommend programming the spectrophotometer to use the shortest configurable inter-well scan interval in kinetic mode.

5. Calculation: For each sample, choose two time points (T_1 and T_2) in the linear phase of the progress curve and calculate the change in absorbance for the time interval ($\Delta OD_{460} = A_2 - A_1$). If sample background control reading is significant, the background control reading may be subtracted from the corresponding sample reading. Apply the ΔOD_{460} to the G6P standard curve to get *B* nmol of substrate reduced by CPR during the reaction time ($\Delta T = T_2 - T_1$).

$$\textbf{NADPH Cytochrome P450 Reductase Activity} = \frac{\boldsymbol{B}}{\boldsymbol{\Delta T \times P}} = \textbf{nmole/min/mg = mU/mg}$$

Where: **B** is the amount of G6P consumed, calculated from the standard curve (nmole)
Δ**T** is the linear phase reaction time (in min.)
P is the amount of protein in the sample (in mg)

CRP activity can also be expressed as mU per ml of sample volume added to the reaction well. For samples with a corresponding sample+inhibitor reading, CPR activity is obtained by subtracting the activity calculated in the presence of inhibitor from the activity in the absence of inhibitor.

CPR Unit Definition: One unit of cytochrome P450 reductase is the amount of enzyme that generates 1.0 µmole of reduced substrate per min. by oxidizing 1.0 µmole NADPH to β-$NADP^+$ at pH 7.7 at 25°C.

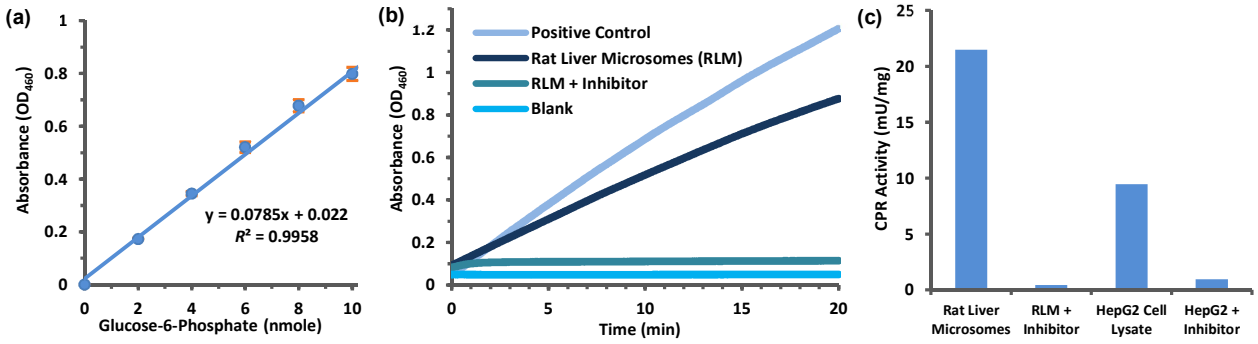


Figure: (a) G6P Standard curve. One mole G6P corresponds to one mole of β-NADP⁺ reduced to NADPH, which subsequently generates one mole of reduced substrate. **(b)** Reaction kinetics of recombinant human CPR (positive control) and rat microsomal CPR (with and without inhibitor). **(c)** Relative CPR activity detected in rat liver microsomes (RLM, 25 µg total protein) and HepG2 cell lysate (40 µg total protein). Assays were performed according to the kit protocol.

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