

ab204704

**Cytochrome P450
Reductase Activity Assay
Kit (Colorimetric)**

Instructions for Use

For rapid, sensitive and accurate detection of Cytochrome P450
Reductase Activity.

This product is for research use only and is not intended for diagnostic
use.

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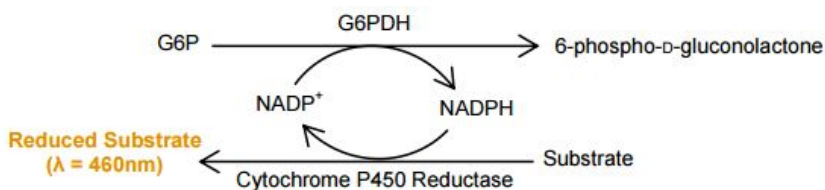
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1. BACKGROUND

Cytochrome P450 Reductase Activity Assay Kit (Colorimetric) (ab204704) couples oxidation of NADPH by cytochrome p450 reductase (CPR) to reduction of a nearly colorless probe into a brightly colored product with an absorbance peak at OD=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).

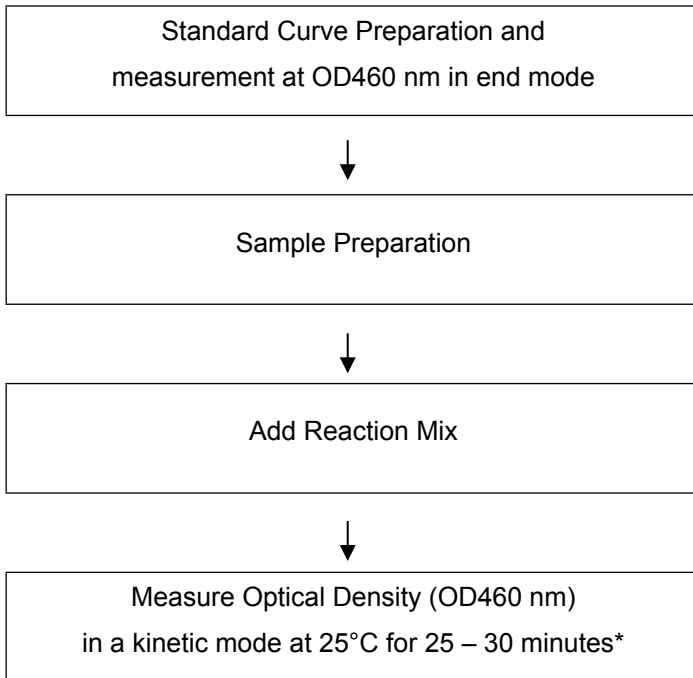
The kit can be used to determine CPR activity in a variety of samples, with a detection limit of ~0.2 mU of CPR activity per reaction.

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.



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.

2. ASSAY SUMMARY



**For kinetic mode detection, incubation time given in this summary is for guidance only.*

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

| Item | Amount | Storage Condition (Before Preparation) | Storage Condition (After Preparation) |
|----------------------------|--------|--|---------------------------------------|
| CPR Assay Buffer | 50 mL | -20°C | -20°C |
| CPR Standard (G6P) | 1 Vial | -20°C | -20°C |
| CPR Inhibitor | 100 µL | -20°C | -20°C |
| NADPH Substrate Mix | 1 Vial | -20°C | -20°C |
| G6P Standard Developer | 1 Vial | -20°C | -20°C |
| Human CPR Positive Control | 1 Vial | -20°C | -80°C |

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (ddH₂O)
- Pipettes and pipette tips
- Microcentrifuge
- Colorimetric microplate reader – equipped with filter OD = 460 nm
- 96 well plate: clear plate with flat bottom
- Heat block or water bath
- Dounce homogenizer

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1. CPR Assay Buffer:

Ready to use as supplied. Warm Assay Buffer to room temperature before use. Store at -20°C.

9.2. CPR Standard (G6P):

Reconstitute standard in 300 μ L ddH₂O to generate a 100 mM (100 nmol/ μ L) G6P stock solution. **NOTE: The CPR Standard (G6P) can be a bit viscous after reconstitution of the lyophilized cake. Use gentle heating at 37°C and sonication to help completely dissolve the G6P.** Aliquot standard solution so that you have enough volume to perform the desired number of assays. Keep on ice while in use. Store at -20°C.

9.3. CPR Inhibitor:

Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use. **NOTE: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C.** Vortex to ensure inhibitor is completely dissolved.

Mix 100 μ L of the 10 mM solution with 900 μ L of CPR Assay Buffer to obtain a final 1 mM inhibitor working solution. Aliquot working solution so that you have enough volume to perform the desired number of assays. Store working solution at -20°C.

9.4. NADPH Substrate Mix:

Reconstitute in 600 μ L CPR Assay Buffer. Aliquot substrate so that you have enough volume to perform the desired number of assays. Avoid repeated freeze/thaw cycles. Store at -20°C. Keep NADPH Substrate Mix on ice while in use

9.5. G6P Standard Developer:

Dissolve in 600 μL dH_2O and pipette up and down to mix thoroughly. Aliquot developer so that you have enough volume to perform the desired number of assays. Store at -20°C .

9.6. **Human CPR Positive Control:**

Reconstitute in 50 μL CPR Assay Buffer by pipetting up and down until fully resuspended. Do not vortex. For best results, we recommend using the reconstituted CPR positive control within one day; however, it can be aliquoted as needed and stored at -80°C . Avoid repeated freeze/thaw cycles. Keep on ice while in use.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted CPR Standard (G6P) solution 1 mM (1 nmol/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 CPR Standard (G6P) solution on ice while in use.

10.1 Prepare 1 mL of 1 mM CPR Standard (G6P) solution by diluting 10 μL of the provided 100 mM CPR Standard (G6P) with 990 μL of CPR assay buffer.

10.2 Using 1 mM CPR Standard (G6P), prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

| Standard # | Volume of Standard (μL) | Assay Buffer (μL) | Final volume standard in well (μL) | End Conc G6P in well (nmol/well) |
|------------|-------------------------|-------------------|------------------------------------|----------------------------------|
| 1 | 0 | 270 | 90 | 0 |
| 2 | 6 | 264 | 90 | 2 |
| 3 | 12 | 258 | 90 | 4 |
| 4 | 18 | 252 | 90 | 6 |
| 5 | 24 | 246 | 90 | 8 |
| 6 | 30 | 240 | 90 | 10 |

Each dilution has enough amount of standard to set up duplicate readings (2 x 90 μL).

11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C . When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- To quantify specific CPR activity in terms of sample protein content, save a small aliquot of the sample and quantify the protein concentration using a Bradford reagent or an equivalent protein assay.

11.1 **Microsomes:**

Isolated microsomes from soft tissues or cultured eukaryotic are preferred as sample type.

Alternatively if not available, a crude microsomal preparation for cells or tissue can be used instead.

11.1.1 Harvest tissue (initial recommendation = 50 mg) or cells (initial recommendation = 5×10^6 cells) for microsomal isolation.

11.1.2 Wash tissue or cells in cold PBS.

11.1.3 Suspend tissue or cells in 500 μL of ice cold CPR Assay Buffer containing protease inhibitor cocktail.

NOTE: Any commercial lysis buffer containing a mild non-ionic detergent can also be used for lysis of cultured cells. In our experience, lysis buffers with similar composition to

the CPR Assay Buffer containing 0.1% Triton X-100 (final concentration $\leq 0.05\%$) do not interfere with the assay.

NOTE: The CPR Assay Buffer does not contain a protease inhibitor cocktail as a stock solution. Add protease inhibitor cocktail as per its instruction in order to prevent sample protein degradation. The protease inhibitor cocktail is not required to run the assay.

- 11.1.4 Homogenize tissue or cells with a Dounce homogenizer sitting on ice, with 10 – 15 passes.
- 11.1.5 Incubate sample on ice for 5 minutes.
- 11.1.6 Centrifuge samples for 5 minutes at 4°C at 1,500 x g using a cold micro centrifuge.
- 11.1.7 Transfer the supernatant to a new pre-chilled microfuge tube.
- 11.1.8 Centrifuge samples for 5 minutes at 4°C at 12,000 x g using a cold micro centrifuge.
- 11.1.9 Collect supernatant and transfer to a clean pre-chilled tube and store on ice. Use lysates immediately to assay CPR activity.

NOTE: We suggest using different volumes of sample to ensure readings are within the Standard Curve range.

12. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
- **Prepare all reagents, working standards, and samples as directed in the previous sections.**

12.1 Standard curve measurement:

- 12.1.1 Set up Standard wells = 90 μ L standard dilutions.
- 12.1.2 To each of the standard wells, add 5 μ L of NADPH Substrate Mix (Step 9.4) and 5 μ L of G6P Standard Developer (Step 9.5) to make the final volume 100 μ L/well. Mix well.
- 12.1.3 Incubate standard wells for at least 30 minutes at room temperature, protected from light.
- 12.1.4 Measure absorbance in a colorimetric microplate reader at OD = 460 nm at 25°C for all of CPR Standard (G6P) wells.

12.2 Set up Reaction wells:

- Sample wells = 5 – 40 μ L samples (adjust volume to 60 μ L/well with CPR Assay Buffer).
- Sample + Inhibitor wells = 5 – 40 μ L samples + 10 μ L inhibitor (adjust volume to 60 μ L/well with CPR Assay Buffer)
- Positive control wells = 5 μ L recombinant CPR + 55 μ L CPR Assay Buffer.
- Background control wells= 5 – 40 μ L samples + 2 μ L inhibitor (adjust volume to 70 μ L/well with CPR Assay Buffer)

The table below shows the reaction wells set up:

| Component | Sample (μL) | Sample + Inhibitor (μL) | Positive Control (μL) | Background Control (μL) |
|------------------|-------------|-------------------------|-----------------------|-------------------------|
| Sample | 5 - 40 | 5 - 40 | 0 | 5 - - 40 |
| CPR Inhibitor | 0 | 10 | 0 | 2 |
| Recombinant CPR | 0 | 0 | 5 | 0 |
| CPR Assay Buffer | up to 60 | up to 60 | up to 60 | up to 70 |

12.3 Reaction Mix:

Prepare 30 μL of Reaction Mix for each reaction:

| Component | Reaction Mix (μL) |
|------------------|-------------------|
| NADPH Substrate | 5 |
| CPR Assay Buffer | 25 |

Mix enough reagents for the number of assays (samples and controls) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X μL component x (Number reactions +1).

12.4 Add 30 μL of Reaction Mix into each well. Mix well.

12.5 Incubate for 5 minutes at room temperature to allow the CPR Inhibitor to bind targets.

12.6 G6P Reaction Solution:

Make a 20 mM G6P Reaction Solution by diluting the 100 mM G6P stock solution (Step 9.2) with CPR Assay Buffer at a 1:5 ratio (for example, mix 20 μL of the 100 mM G6P stock with 80 μL CPR Assay Buffer to yield 100 μL of 20 mM G6P reaction solution).

12.7 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.

- 12.8 Immediately measure absorbance at OD460 nm in kinetic mode for 25 - 30 minutes at 25°C

NOTE:

- a) *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.*
- b) *Incubation time depends on the CPR Activity in the samples. We recommend measuring OD in a kinetic mode, and choosing two time points (T_1 and T_2) in the linear range (OD values A_1 and A_2 respectively) to calculate the CPR activity of the samples. The Standard Curve can be read in end point mode (i.e. at the end of incubation time).*

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 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 ($\Delta T = T_2 - T_1$).

$$\text{NADPH Cytochrome P450 Reductase Activity} = \left(\frac{B}{\Delta T \times P} \right) = \text{nmol/min/mg} = \text{mU/mg}$$

Where:

B = Amount of G6P consumed, calculated from the standard curve (nmole).

ΔT = Linear phase reaction time (min).

P = Amount of protein in the sample (mg).

13.2 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 μ mole of reduced substrate per min. by oxidizing 1.0 μ mole NADPH to β -NADP⁺ at pH 7.7 at 25°C.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

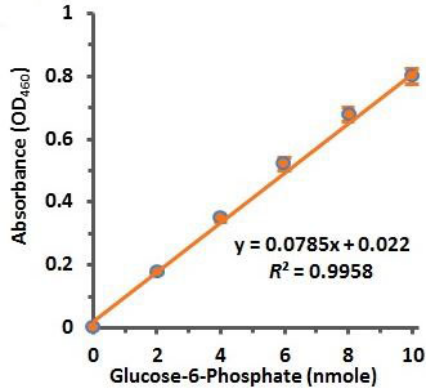


Figure 1. Typical G6P Standard calibration curve. One mole G6P corresponds to one mole of β -NADP⁺ reduced to NADPH, which subsequently generates one mole of reduced substrate.

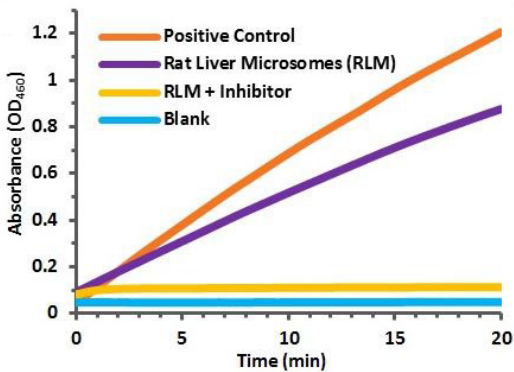


Figure 2. Reaction kinetics of recombinant human CPR (positive control) and rat microsomal CPR (with and without inhibitor).

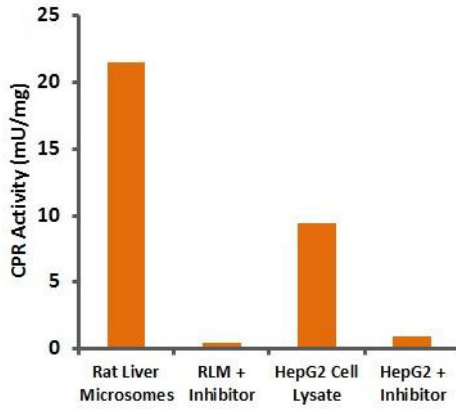


Figure 3. Relative CPR activity detected in rat liver microsomes (RLM, 25 μ g total protein) and HepG2 cell lysate (40 μ g total protein).

15. QUICK ASSAY PROCEDURE

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare standard, positive control and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare appropriate standard curve.
- Add 5 μL NADPH substrate + 5 μL G6P Standard developer to standard wells (90 μL standard). Incubate 30 min RT. Measure absorbance at OD = 460 nm.
- Prepare samples in duplicate.
- Set up plate for samples, sample + CPR Inhibitor, Positive control and background wells as follows:

| Component | Sample (μL) | Sample + Inhibitor (μL) | Positive Control (μL) | Background Control (μL) |
|------------------|--------------------------|--------------------------------------|------------------------------------|--------------------------------------|
| Sample | 5 - 40 | 5 - 40 | 0 | 5 - 40 |
| CPR Inhibitor | 0 | 10 | 0 | 2 |
| Recombinant CPR | 0 | 0 | 5 | 0 |
| CPR Assay Buffer | up to 60 | up to 60 | up to 60 | up to 70 |

- Prepare CPR Reaction Mix (Number reactions + 1).

| Component | Reaction Mix (μL) |
|------------------|--------------------------------|
| NADPH Substrate | 5 |
| CPR Assay Buffer | 25 |

- Add 30 μL of CPR Reaction Mix to each well.
- Add 10 μL of 20 mM G6P solution to each well except background control). Mix well.
- Immediately measure absorbance at OD=460 nm at 25°C for 258 – 30 minutes in a kinetic mode.

16. TROUBLESHOOTING

| Problem | Cause | Solution |
|--|--|---|
| Assay not working | Use of ice-cold buffer | Buffers must be at room temperature |
| | Plate read at incorrect wavelength | Check the wavelength and filter settings of instrument |
| | Use of a different 96-well plate | Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate |
| Sample with erratic readings | Samples not deproteinized (if indicated on protocol) | Use PCA precipitation protocol for deproteinization |
| | Cells/tissue samples not homogenized completely | Use Dounce homogenizer, increase number of strokes |
| | Samples used after multiple free/ thaw cycles | Aliquot and freeze samples if needed to use multiple times |
| | Use of old or inappropriately stored samples | Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use |
| | Presence of interfering substance in the sample | Check protocol for interfering substances; deproteinize samples |
| Lower/ Higher readings in samples and Standards | Improperly thawed components | Thaw all components completely and mix gently before use |
| | Allowing reagents to sit for extended times on ice | Always thaw and prepare fresh reaction mix before use |
| | Incorrect incubation times or temperatures | Verify correct incubation times and temperatures in protocol |

| Problem | Cause | Solution |
|--|---|--|
| Standard readings do not follow a linear pattern | Pipetting errors in standard or reaction mix | Avoid pipetting small volumes (< 5 μ L) and prepare a master mix whenever possible |
| | Air bubbles formed in well | Pipette gently against the wall of the tubes |
| | Standard stock is at incorrect concentration | Always refer to dilutions on protocol |
| Unanticipated results | Measured at incorrect wavelength | Check equipment and filter setting |
| | Samples contain interfering substances | Troubleshoot if it interferes with the kit |
| | Sample readings above/ below the linear range | Concentrate/ Dilute sample so it is within the linear range |

17. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure.

- RIPA buffer – it contains SDS which can destroy/decrease the activity of the enzyme.

Technical Support

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