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# Measurement of cytochrome P450 and NADPH–cytochrome P450 reductase

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## Abstract

Cytochrome P450 (P450) enzymes are important in the metabolism of steroids, vitamins, carcinogens, drugs and other compounds. Two of the commonly used assays in this field are the measurements of total P450 and NADPH–P450 reductase in biological preparations. A detailed protocol is presented for the measurement of P450 by its spectral properties, along with a protocol for measuring NADPH–P450 reductase by its NADPH–cytochrome *c* reduction activity. Each assay can be completed in 5–10 min. Detailed explanations for the rationale of particular sequences in the protocols are provided, along with potential confounding problems.

## INTRODUCTION

Cytochrome P450 (P450) enzymes have been studied for more than 50 years. They have important roles in the metabolism of steroids, fat-soluble vitamins, carcinogens and drugs. In drug metabolism, three-fourths of the enzymatic reactions are catalyzed by P450s[1]. Of the 57 human P450 (*CYP*) genes, 5 P450 proteins account for ~95% of the P450 drug oxidations[1–3].

On account of the above-mentioned roles of P450 enzymes and activities in various fields including drug metabolism, endocrinology, chemical carcinogenesis and microbiology[4], it is of interest to be able to quantify P450 enzymes and also the accessory protein, NADPH–P450 reductase, which is necessary for catalytic activity. Studies of P450 enzymes have led to considerable progress in the mechanisms of gene regulation, catalysis and function of these important hemeproteins, much of which has been applied in the area of pharmaceutical development[2]. Many of the assays of P450 activity involve highly sensitive measurements of the oxidation of drugs or surrogate compounds (e.g., see articles in this issue by Sohl *et al.*[5] and Cheng *et al.*[6]). Sensitive methods are available for determining levels of the expression of individual P450 mRNAs (e.g., reverse transcription-PCR) and proteins (immunochemical). However, in many cases, the assays of total P450 (an assay encompassing all forms in a system) and NADPH–P450 reductase are preferred, or sometimes only one of the two measurements. The principle of the P450 spectral assay (option A) is that the ferrous form of the hemeprotein reacts with carbon monoxide (CO) to form a complex that specifically produces a spectrum with a wavelength maximum at ~450 nm, owing to the signature cysteine thiolate axial ligand to the heme iron in these proteins. The original P450 spectrum was reported in 1958 (ref. 7), and the extinction coefficients



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were developed by Omura and Sato[8,9]. The method measures all of the P450s in a particular biological sample and this is used as a parameter. The assay is also used to characterize recombinant or purified P450 preparations in the context of the stoichiometry of active P450 per unit protein. A useful aspect is that the loss of activity of P450 enzymes is associated with a loss of the 450-nm spectrum described here and conversion to a form with a wavelength maximum at 420 nm[10]. Thus, the assays serve as a check on the integrity of the enzyme, which is useful in routine research activities.

As alluded to above, alternatives to the assay of P450 proteins include the use of mRNA and immunochemical assays of individual (or sets of) P450s. Neither of these is a guide to the total amount of P450, nor to the presence of active P450. Measuring only heme absorbance (or total heme) does not discern P450 from other hemeproteins or provide information regarding how much is catalytically active.

The NADPH–P450 reductase assay (option B) is based on the use of the electron acceptor surrogate cytochrome *c*, because this acceptor does not reoxidize in air and provides a convenient laboratory assay, as do several dyes mentioned later. Immunochemical (or mRNA) assays do not provide information regarding whether the protein is active. Both a P450 and the NADPH–P450 reductase are necessary for almost all P450 reactions (except for the five—in humans—catalyzed by mitochondrial P450s[2]). Thus, measuring both spectral P450 and NADPH–cytochrome *c* reduction can be used as checks on the integrity of a biological system.

Our recent experience is that many researchers have some confusion regarding basic aspects of both of these assays, and we are of the opinion that a detailed explanation and protocols are in order.

## Basic principles

The predominant method of assaying total P450 involves what is termed the ferrous CO versus ferrous difference spectrum. Only the reduced (ferrous) form of P450 binds CO, as is the case with hemoglobin and other hemoproteins. Thus, the principle is that the reference cuvette will contain only ferrous P450 (reduced artificially with the reducing salt sodium dithionite, $Na_2S_2O_4$), and the sample cuvette will contain the same ferrous P450 bound to CO. The extinction coefficient (91,000 $M^{-1}$ $cm^{-1}$ at 450 nm in this difference spectrum) appears to be generally valid for most P450s.

The flavoprotein NADPH–P450 reductase donates electrons to P450, but the assay does not measure the activity directly because of the propensity of reduced P450 to react with air, except in the presence of excess $Na_2S_2O_4$, as in the assay prescribed here. This is also a problem, although to a lesser degree, with cytochorme $b_5$, which is also reduced by this flavoprotein[11]. Therefore, as is the case with many mitochondrial electron transport chain enzymes, the most convenient approach is to couple the reaction to air-stable dyes or other redox components, e.g., cytochrome *c*. In this assay, the reductase accepts electrons from the biological hydride donor NADPH and transfers these (one at a time) to cytochrome *c*[12]. Although the assay does not distinguish against a proteolytically cleaved form of NADPH–P450 reductase that binds and reduces cytochrome *c* but not P450s (because of the loss of the membrane-anchoring N-terminal tail that facilitates interaction between the two proteins)[13], proteolytic cleavage in liver microsomes (and many other tissues) is minimal and the cytochrome *c* reduction rate is generally accepted as a good surrogate of the NADPH–P450 reduction activity (although not occurring at the same rate).

## Experimental design

Before beginning these assays, it is critical to be sure that an appropriate recording spectrophotometer is available for wavelength scanning in the P450 spectral assays or in a kinetic mode in the cytochrome *c* reduction assay. In this laboratory, we routinely use a modified OLIS/Aminco DW2a instrument (On-Line Instrument Systems), which has excellent optical properties because of the positioning of the photomultiplier tube. Other commercial instruments are also adequate, particularly if turbidity is reduced by solubilizing any turbid samples with detergents. In principle, it is possible to use certain plate reading instruments for these measurements[14].

The choice of buffers for preparing the biological samples is not critical, except that care should be taken in preparing microsomal samples to avoid lipid peroxidation and proteolytic artifacts[15]. The assays described here are routinely carried out in potassium phosphate buffers (pH 7.4–7.7), although other buffers could be used. The presence of the high concentration of glycerol is important for P450 stability. The cholate and non-ionic detergent used in the P450 spectral assays serve to decrease the turbidity. The ionic strength is not an issue in the case of the P450 spectral assay, but the NADPH–cytochrome *c* reduction activity increases with increasing ionic strength and 0.3 M of potassium phosphate is optimal[16].

A concentration of 0.5–5 μM P450 is preferred in the spectral assay. Assays with less material can be carried out, with care. In the reductase assay, the amount of material should be sufficient to yield a change of at least 0.02 absorbance unit per min, i.e., 1 nmol of cytochrome *c* reduced per min per ml. A rate faster than 0.4 absorbance units per min (i.e., 20 nmol of cytochrome *c* reduced) is often difficult to quantify.

The calculations are generally straightforward after the data are recorded. However, in the case of low activity in either assay, the estimates can become more problematic. The main complication with the spectral assay is a high concentration of a peak at 420 nm and an uneven baseline[17].With the cytochrome *c* reduction assay, a low activity can be problematic. It is necessary to record a change in $A_{550}$ (1–3 min) before adding the NADPH. In some (sensitive) cases, it may be necessary to subtract this rate from the rate recorded after NADPH addition.

The major applications of these methods historically involve measurements in microsomes prepared from tissues of laboratory animals or humans. More recently, the methods were used with heterologous expression systems, particularly *Escherichia coli* and baculovirus-based systems[18]. With some expression systems, the concentrations are too low for measurements (e.g., COS7 cells). Measurements of P450 spectra are considered critical in establishing the validity of physical and biochemical studies carried out with novel P450s, e.g., those isolated from microorganisms or plants, in the context of defining protein integrity. Similarly, the NADPH–cytochrome *c* reduction activity is used as a guide to function, with appropriate caveats.

One of the limitations of these assays is that unusually turbid samples cannot be used. Some spectrophotometers are better for handling turbidity (light scattering), particularly if the cuvettes are positioned close to the photomultiplier tube (see above). Another limitation is that samples having low level of P450 in the presence of high concentrations of cytochrome P420, hemoglobin, methemoglobin or other hemoproteins are problematic because of interference. Thus, liver samples (and sometimes the kidney) are usually not problematic, but tissues such as from the lung and brain are highly problematic.

With regard to the reductase assay, a limitation is that the microsomes prepared from tissue homogenates (or bacterial extracts, in the case of heterologous expression) may contain other reductases with the ability to transfer electrons to cytochrome *c*.

As pointed out, both of these assays should be carried out in the absorbance range of 0.05–1.0 in order to have sufficient absorbance to measure accurately, but not so high as to be out of the operating range of the instruments. In some cases, it is possible to accurately collect reliable data at > 1.0 absorbance units (e.g., Cary 14/17 instruments) because of the stray light characteristics.

### (A) P450 spectral assay—

- The order of the addition of the reagents is important. CO should be added to the sample cuvette before $Na_2S_2O_4$. Dithionite is unstable and reacts with oxygen to generate superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$), which are deleterious to P450 and its heme prosthetic group[19]. Thus, adding dithionite to a CO-saturated sample causes the P450 to be trapped as the reduced-CO complex as soon as reduction begins. Because of the properties of the *d*-orbital electrons, only the ferrous forms of hemoproteins bind CO.

- One of the problems with this assay is that it does not readily distinguish between (cytochrome) P420, a conglomerate term used for forms of P450 that have lost the characteristic thiol ligand (protein cysteine)[10], and contaminating hemeproteins that often show spectral peaks at 420 nm. Notorious problems are seen with most extrahepatic mammalian tissues and also some heterologous expression vector systems, e.g., *E. coli* systems showing low expression levels[17,20,21].

  One approach to recording P450 spectra in the presence of contaminating hemoglobin is to alter the system to record an ‘oxidized CO’ versus reduced CO difference spectrum. Hemoglobin is in the ferrous state and will bind CO, so theoretically if the only contaminant is oxyhemoglobin, the complex will show the same spectrum in both cuvettes and not interfere. Under these conditions, an extinction coefficient ($\Delta\varepsilon_{450–490}$) of 106,000 $M^{-1}$ $cm^{-1}$ has been calculated, which should be substituted (for the normal 91,000 $M^{-1}$ $cm^{-1}$ because of the correction for the oxidized hemoprotein instead of the reduced)[20].

  The problem is more pronounced with methemoglobin or other *ferric* hemoproteins, as they will not bind CO unless reduced and therefore look very similar to cytochrome P420. One approach that has been suggested is the use of ascorbate, which is reported to reduce methemoglobin but not cytochrome P420[21]. This approach has been only partially successful in our own experience; its effectiveness in working with bacterial hemoprotein systems is unknown.

- Reduction to a stable spectrum usually occurs rapidly, within a few 100-nm scans (≤5 min). However, in our experience, some particular P450s are prone to slow reduction or poor spectra. Some of the plant P450s have weak affinity for CO and yield poor spectra[22,23]. Some mammalian P450s, e.g. human P450 1A2[24], are reduced slowly by dithionite (but rapidly by NADPH–P450 reductase). The problem appears to be access of the salt dithionite to the heme of this P450. To remedy the problem of slow reduction, a mediator dye is added to the P450 sample before it is divided into the two cuvettes. In our experience, 0.5–2 μM safranine T serves as a useful mediator[24]. The oxidized dye is red, but the reduced dye is colorless and, because this is present in both cuvettes, does not interfere with the spectra. The presence of safranine T can reduce the time required for reduction from > 20 min to ~3 min (ref. 24).

- We and others have encountered several unusual problems with P450 19A1, the P450 that converts androgens to the aromatic (A-ring) estrogens ('aromatase'), which are still unexplained. Under some purification conditions, the protein can be isolated, but the enzyme has lost heme[25,26]. In other cases, the isolated P450 clearly has a low-spin iron heme spectrum ($\lambda_{max}$ = 418 nm) but does not bind CO when dithionite is added[26]. However, even in the absence of the typical P450 spectrum, the isolated protein (when mixed with NADPH–P450 reductase) had catalytic activity[25]. These observations led earlier workers in the field to question whether the aromatase is actually a P450[25]. (The question appears to have been resolved unambiguously with the structure determination of the protein isolated from the human placenta[27].) Recently, we have found ligands that, when used during protein chromatography, stabilize the enzyme and allow it to be obtained in a form that has rather normal spectral properties, especially in binding CO. The approach of using safranine T as a mediator dye (see above) is also useful in this case.

### (B) NADPH–P450 reductase assay—

- Cytochrome *c* is readily available and is the most popular electron acceptor used in this assay (historically, the horse heart cytochrome *c* has been used because of ready availability, although other species can be substituted). However, several dyes and electron acceptors have also been used, including dichlorophenol indophenol[28], ferricyanide[29] and tetrazoliums[30]. These have lower extinction coefficients or involve a decrease in absorbance (instead of an increase). Stocks of cytochrome *c* can be prepared and frozen between use without problems.
- With mammalian microsomal preparations, there is little interference. NADH–cytochrome $b_5$ reductase (and cytochrome $b_5$) is present and can transfer electrons to cytochrome *c*, but this reductase does not use NADPH efficiently. However, in bacteria used for heterologous expression of P450s, there is potential for other reductases that can transfer electrons from NADPH to cytochrome *c*. Some of the systems are not membrane bound and do not interfere if NADPH–P450 reductase is expressed in the membranes. One means of blocking these extraneous reductases is to include 1 mM of KCN, which has no effect on the NADPH–P450 reductase[15,18].
- Under these conditions, 1 nmol of NADPH–cytochrome P450 reductase will reduce about 3,000 nmol of cytochrome *c* per min at 23 °C[16,18,31]. Accordingly, the rate can be reported as nmol of cytochrome *c* reduced per min by a certain protein concentration or a conversion to mol of NADPH–cytochrome P450 reductase can be made.

## MATERIALS

### REAGENTS

- $Na_2S_2O_4$. Prepare a small vial (~ 5 g) from the stock and keep tightly capped and dessicated at ambient temperature (i.e., ~23 °C). ▲ **CRITICAL** Do not pre-prepare the solutions because the salt readily oxidizes in air.
- Safranine T (also termed safranine O) (Sigma-Aldrich, cat. no. S8884). This can be dissolved in the buffer and stored at 4 °C for several months.
- Glycerol (Fisher, cat. no. G33-4)
- Sodium cholate (Sigma-Aldrich, cat. no. C6445)
- Non-ionic detergent: choice of Triton N-101 (reduced form, Sigma-Aldrich, cat. no. 303135) or Tergitol NP-40 (Sigma-Aldrich, cat. no. NP405)

- Potassium phosphate (Fisher, cat. no. P285 (monobasic) and P288 (dibasic)); dissolve 25.9 g of monobasic and 141.0 g of dibasic in 1 liter of $H_2O$ to prepare a 1.0-M solution at pH 7.4 (Note: add the phosphate to the $H_2O$ and not vice versa)
- EDTA (Fisher, cat. no. S311-100)
- Cytochrome c, from horse heart (Sigma, cat. no. 7752)
- NADPH (Sigma, cat. no. N4505)
- Carbon monoxide (CO) compressed gas (Specialty Gases of America). **! CAUTION** Highly toxic! Can be fatal at high doses! Store the cylinder and use in a well-ventilated fume hood. The tank should be equipped with a two-stage regulator, attached to a flexible tubing with a Pasteur pipette attached (and a pinch clamp on the tubing).

### EQUIPMENT

- Cuvettes, generally 1-ml capacity, 1 cm (either glass or plastic), e.g., Bio-Rad disposable cuvettes (Bio-Rad, cat. no. 223-9955)
- Spectrophotometer. If turbidity is an issue, an instrument with a strong light source and a photomultiplier tube close to the cells is preferred. The instrument must be capable of scanning wavelength
- Optional Add-A-Mixer (or ‘plumper’) device (NSG Precision Cells, cat. no. P68)

## PROCEDURE

▲ **CRITICAL** All assays should be carried out at ambient temperature (i.e., ~23 °C). Buffers and solutions should be stored at 4 °C when not in use.

**1|** P450 activity can be estimated using option A for total P450 (an assay encompassing all forms in a system) or option B for the NADPH–P450 reductase.

### (A) Measurement of total P450 ● TIMING 5–10 min

**i.** Dilute the biological sample containing P450 into 2.0 ml of 100 mM of potassium phosphate buffer (pH 7.4–7.7) containing 1.0 mM of EDTA, 20% glycerol (vol/vol), 0.5% sodium cholate (wt/vol) and 0.4% (wt/vol) non-ionic detergent (e.g., Triton N-101) in a small test tube.

**ii.** Mix the contents by capping the test tube with Parafilm (or a cap) and inverting/re-inverting several times (do not mix vigorously or with a vortex device).

**iii.** Divide the sample into two 1 ml cuvettes (glass or disposable plastic), using a glass Pasteur pipette and a bulb.

**iv.** If there is any moisture in the cuvettes from a previous experiment, place the Parafilm over the tops and mix by gentle inversion, as in Step 1A(ii) above.

**v.** Place the two cuvettes in the spectrophotometer and record a baseline between 400 and 500 nm. Most modern spectrophotometers will have a baseline correction mode; use this to ‘flatten’ the baseline.

**? TROUBLESHOOTING**

**vi.** Remove the sample cuvette from the spectrophotometer and (in the fume hood) slowly bubble CO gas through this sample from a clean Pasteur pipette (be sure that the CO line is filled with CO before starting). The rate should be about one

bubble per second, with the end of the Pasteur pipette inserted to the bottom of the cuvette. Count ~60 bubbles. Ensure that no liquid is displaced onto the sides of the cuvette (if so, wipe off).

**! CAUTION** CO is highly toxic! Can be fatal at high doses! Use in a fume hood.

**vii.** Remove the reference cuvette from the spectrophotometer.

**viii.** Add ~1 mg of solid $Na_2S_2O_4$ to both cuvettes, which is, in practice, the amount that can be held on the tip of a small spatula used for this purpose. An attempt should be made to add approximately the same amount of dithionite to both cuvettes.

**ix.** Place pieces of Parafilm over the tops of the two cuvettes and invert (without vigorous shaking) to dissolve $Na_2S_2O_4$ and mix the contents. Using a tissue, wipe off any liquid that might be on the outside of the cuvettes.

**x.** Place the cuvettes back into the spectrophotometer. Ensure that these are in the same positions as originally used (some instruments may have room for 'play' in the fittings).

**xi.** Record the spectrum between 400 and 500 nm several times, over a period of a few minutes, preserving the spectra in the instrument or on chart papers.

**? TROUBLESHOOTING**

**xii.** When the size of the peak near 450 nm has stopped increasing, terminate the analysis.

**xiii.** To begin the calculations, read the absorbance at 450, 490 and 420 nm.

**xiv.** Use the following formula to calculate the cytochrome P450 concentration (see Fig. 1 and Box 1):

$$(\Delta A_{450} - \Delta A_{490})/0.091 = \text{nmol of P450 per ml}$$

**xv.** If the baseline spectrum showed a difference between the absorbances at 450 and 490 nm, then use this correction as follows:

$$[(A_{450} - A_{490})_{observed} - (A_{450} - A_{490})_{baseline}]/0.091 = \text{nmol P450 of per ml}$$

**xvi.** Estimate the content of cytochrome P420, which consists of denatured forms of P450, using the following formulas:

$$\text{nmol of P450 per ml (from Step 1A(xiv))} \times (-0.041) = (\Delta A_{420} - A_{490})_{theoretical}$$

$$[(\Delta A_{420} - A_{490})_{observed} - (A_{450} - A_{490})_{theoretical} - (\Delta A_{420} - A_{490})_{baseline}]/0.110 = \text{nmol of cytochrome P420 per ml}$$

### (B) Measurement of NADPH–cytochrome *c* reduction activity ● TIMING 5 min

**i.** Pipette 80 μl of a 0.5-mM solution of the horse heart cytochrome *c* (in 10 mM of potassium phosphate buffer, pH 7.7) into a 1-ml of glass or plastic cuvette (path length of 10 mm).

**ii.** Add an aliquot of the biological sample to be tested.

**iii.** Pipette 0.3 M of potassium phosphate buffer (pH 7.7) to reduce the total volume in the cuvette to 0.99 ml. Mix the components using an 'Add-A-Mixer' (or a 'plumper' device) or covering the cuvette with Parafilm and inverting several times.

**iv.** Set the spectrophotometer to 550 nm, in the kinetic mode. Preferably, the slit width in the spectrophotometer should be 1.0 nm (because the spectral band is rather sharp). The full-scale absorbance should be 1.

**v.** Record a baseline rate for 2–3 min (at ambient temperature).

**vi.** Add 10 μl of a 10-mM NADPH solution. This addition can be done using an 'Add-A-Mixer' (or a 'plumper') device; less 'dead time' will elapse between the time the reaction is started and when observation begins. Alternatively, remove the cuvette from the chamber for addition of the NADPH; it is necessary to place Parafilm over the cuvette and mix it by inverting before placing it back in the spectrophotometer.

▲ **CRITICAL STEP** Prepare the NADPH solution daily in $H_2O$ and store on ice when not in use.

**vii.** Record $A_{550}$ with the spectrophotometer as a function of time (about 3 min).

**? TROUBLESHOOTING**

**viii.** When the plot of $A_{550}$ versus time is no longer linear, terminate the assay.

**ix.** Calculate the rate of reduction of cytochrome *c* using the formula below (see Fig. 2 and Box 2) for example.

$$\frac{\Delta A_{550}/\text{min}}{0.021} = \text{nmol of cytochrome } c \text{ reduced per min}$$

● **TIMING**

Steps 1A(i–xii), Measurement of total P450: 5–10 min per sample

Steps 1B(i–viii), NADPH–cytochrome *c* reduction activity: 5 min per sample (some instruments can do multiple assays simultaneously)

**? TROUBLESHOOTING**

Troubleshooting advice can be found in Table 1.

## ANTICIPATED RESULTS

In the rat liver microsomes, the concentration of total P450 will generally be in the range of 0.5–1 nmol of P450 per mg protein (the value increases when the animals are treated with certain compounds such as barbiturates)[32]. In the human liver, the value is somewhat lower, depending on the sample quality, but generally 0.2–0.5 nmol per mg of protein. A representative value for the activity of NADPH–cytochrome *c* reduction in rat liver microsomes is 100–400 nmol of cytochrome *c* reduced per min per mg protein, which corresponds to ~0.033 to 0.13 nmol of NADPH–P450 reductase per mg protein[16,32].

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## BOX 1 | SAMPLE CALCULATIONS FOR P450 SPECTRAL ASSAY[7]

For the example presented in Figure 1 where a 1/10 dilution was used (baseline was not corrected for in this example):

$\frac{0.278}{0.091}$ = 3.06 μM P450; × 10 = 30.6 μM P450

Theoretical $A_{420}-A_{490}$ = 3.06 × −0.041 = −0.125

Observed $A_{420}-A_{490}$ = 0.113

(−0.113) − (−0.125) = 0.013

0.013/0.110 = 0.11 μM P420, × 10 = 1.1 μM of P420

## BOX 2 | SAMPLE CALCULATIONS FOR NADPH–CYTOCHROME *C* REDUCTION ASSAY

For the results shown in Figure 2, over the time period of 0.6 min (indicated by arrows, 1.8–2.4 min), the absorbance increased from 0.036 to 0.191 (net gain 0.155).

Thus, 0.155 $\Delta A_{550}$/min/0.6 min/0.021 $mM^{-1}$ $cm^{-1}$ = 12 nmol of cytochrome *c* reduced in the cuvette. As only 5 μl of the reductase preparation was used, the activity of the stock reductase is 12/0.005 ml = 2,400 nmol of cytocchrome *c* reduced per ml of enzyme stock.

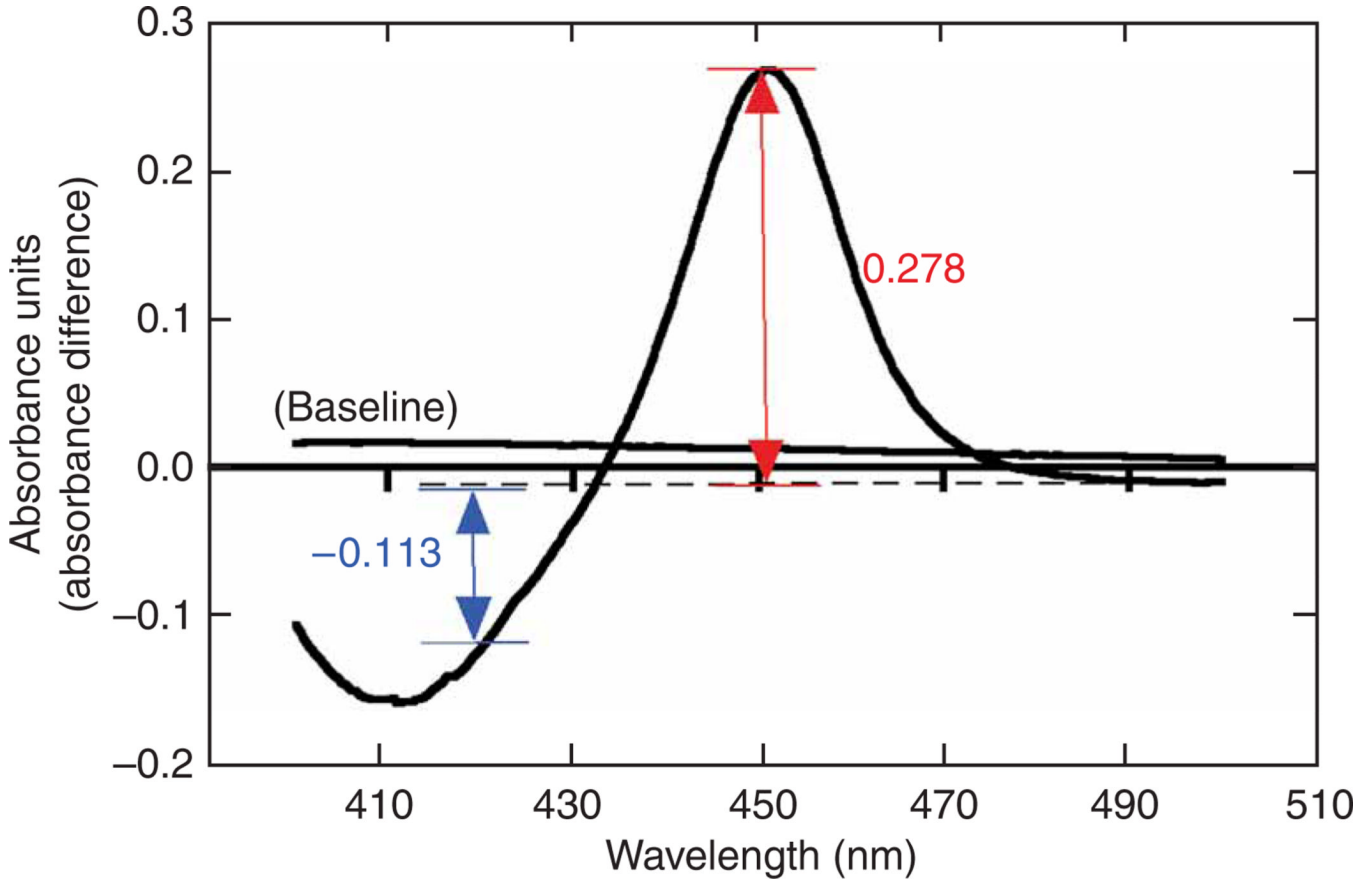


**Figure 1.**

Ferrous · CO versus ferrous difference spectrum used for the quantitation of a preparation of (*E. coli*) recombinant P450 2C9. The P450 concentration was 30.6 μM and the sample was diluted 10-fold for making the measurements. See Box 1 for calculations[9].

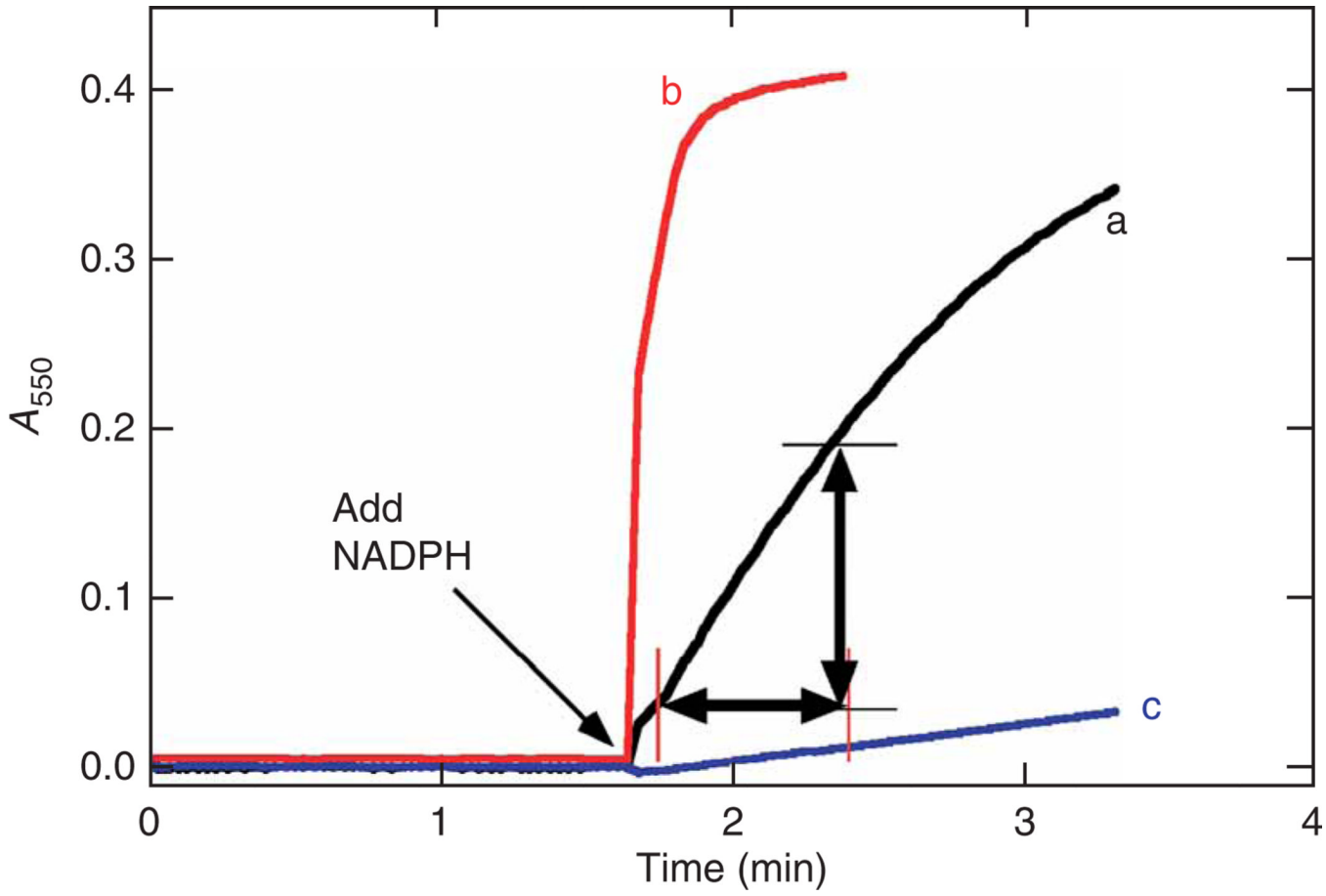


**Figure 2.**

NADPH–cytochrome *c* reduction assay and sample calculations. In this example, this assay was performed using aliquots of recombinant rat NADPH–P450 reductase expressed in *E.coli* and the pre-incubation time (before adding NADPH, as indicated with the arrow) was 1.6 min. See Box 2 for calculations done using trace a, obtained using 5 μl of a purified preparation. In trace b, an excess amount of reductase was added and the reaction was also initiated with NADPH addition; because of the high concentration of reductase, the rate is too fast. In trace c, the amount of reductase was lower than optimal and the reaction was initiated by the addition of NADPH. The results are not optimal due to a lower signal-to-noise ratio, although a rate could be calculated.

## TABLE 1

Troubleshooting guides for spectral P450 and NADPH–cytochrome *c* reduction assays.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 1A(v) | Cannot balance cuvettes in the spectrophotometer | Sample is too turbid | Dilute the sample; need an instrument capable of handling turbidity |
| 1A(xi) | No peak | Dithionite decomposed | Use fresh vial of dithionite (check by adding to a solution of safranine T, which should rapidly lose color) |
| | | CO not introduced | Purge CO line by opening the valve, to purge air, then fill the cuvette with CO again |
| | Very slow reduction | Dithionite does not contact P450 well | Repeat with 1–2 μM of safranine T in the sample (see text) |
| 1B(vii) | No change in $A_{550}$ | NADPH is decomposed | Prepare fresh NADPH |
| | | Concentration of reductase is too low | Add more of the biological sample |