A whole cell assay for spectroscopic measurement of recombinant cytochrome P450 expression in bacteria

Application Note

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Introduction

Cytochrome P-450 refers to a family of enzymes that are of central importance in catalysing the hydroxylation of a variety of organic compounds[1,2] (Figure 1).

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Figure 1. Stoichiometry of P450-catalyzed reaction

Cytochrome P-450s play an important role in the biosynthesis and degradation of compounds such as steroids, prostaglandins and fatty acids and are also involved in the oxygenation of many xenobiotics (foreign compounds) such as therapeutic drugs, carcinogens and chemical pollutants[2,3]. These reactions often determine the metabolic fate of the compound in question. Some cytochrome P-450-dependent metabolic steps detoxify xenobiotics, while other steps bioactivate them (i.e., convert them into more toxic metabolites). Most environmental carcinogens are actually "pro-carcinogens" which, although intrinsically unreactive, are metabolized to reactive species that can induce DNA mutations.

Bacteria generally do not carry out bioactivation processes. Therefore, bacterial mutation assays reproduce mammalian metabolic pathways by incorporating “S9” (rodent liver homogenate). Since 1995, bacterial mutagenicity tester strains have been developed which express recombinant human enzymes and can bioactivate carcinogens[4]. This approach obviates the need for rodent S9 and makes possible short-term mutagenicity assays which are much more predictive of human enzymology and metabolism. This approach can also be applied to analysis of the structure and function of human cytochrome P-450.

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Cytochrome P-450 enzymes are about 500 amino acid residues in length, including an N-terminal endoplasmic reticulum (ER) retention signal. Removal of the N-terminal ER retention signal has been shown to markedly increase expression of cytochrome P-450 in bacterial cells without altering catalytic activity. In addition, co-expression of the electron supplying NADPH-cytochrome reductase increases cytochrome P-450 activity in *E. coli* by a large factor[5], allowing recombinant cytochrome P-450 enzymes to be expressed at levels up to hundreds of nmol of protein per L of bacterial culture[6].

Spectrophotometric determination of cytochrome P-450 concentration relies on measuring the optical difference spectrum between reduced enzyme (reference) and reduced-carbon monoxide complex (sample). This procedure is frequently carried out on tissue homogenates or microsomal preparations, which are quite turbid and so require the use of a spectrophotometer that performs accurately and reliably at high absorbance values. This study compares the use of whole bacterial cells to plasma membrane vesicles for the determination of cytochrome P-450 levels in a recombinant bacteria. Carrying out the assay on whole bacterial cells demands accurate measurement of absorbance changes of about 0.02 units, against a background optical density (due mainly to light scattering by the cells) of about 3 units. The Agilent Cary 300 UV-Vis gave excellent results even under these demanding conditions.

Materials and methods

Equipment:

(For part numbers please refer to Reference 9)

- Agilent Cary 300 UV-Vis Spectrophotometer
- Standard 10 mm pathlength rectangular quartz cuvette
- Cary WinUV Bio Package Software

Bacterial cultures:

Strain DJ4309 is an *Eschericia coli* strain containing a recombinant plasmid for expression of cytochrome P-450. Grow 50 mL bacterial culture in Terrific Broth[7] supplement with appropriate antibiotics for plasmid maintenance, 1 mM IPTG for induction of cytochrome P-450 expression from the lac promoter and 0.5 mM delta-ALA (a heme precursor available from Sigma) and 2.5 mL/L trace elements for high-level cytochrome P-450 expression.

Trace element mix is prepared as follows:

To make 100 mL

$FeCl_3.6H_2O$	2.7 g
$ZnCl_2.4H_2O$	0.2 g
$CoCl_2.6H_2O$	0.2 g
$Na_2MoO_4.2H_2O$	0.2 g
$CaCl_2.2H_2O$	0.1 g
$CuCl_2.6H_2O$	0.13 g
H_3BO_3	0.05 g
Concentrated HCl	10 mL

Make up to 100 mL with H_2O

Bacterial cultures are grown for 24 h at 30 °C with vigorous shaking (350 rpm) in 250 mL Erlenmeyer flasks until A_{600} is approximately 7.0.

Sample preparation:

Prepare 100 mL sodium phosphate buffer, 0.1 M, pH 7.4, plus 20% glycerol (PBG). Use this buffer for all subsequent washes and resuspensions.

1. Whole cells

Before centrifuging, weigh the empty centrifuge tube. Centrifuge 12 mL culture (3500 rpm, 5 min, 4 °C), resuspend the cell pellet in 1 mL PBG, recentrifuge and discard the supernatant. Calculate the pellet wet weight by difference. The final expression level can be expressed per gram wet weight of cells. Resuspend the pellet in 1 mL PBG in a 15 mL glass conical-bottom centrifuge tube, add PBG to 6 mL total volume and vortex (to give two-fold concentrated culture). The samples may now be stored on ice until spectroscopic measurement.

2. Membrane vesicles

Plasma membrane vesicles were prepared by French press disruption of spheroplasts[8].

Instrument and software parameters:

The Cary WinUV Scan application was set to record the cytochrome P-450 spectrum by scanning from 500 nm to 400 nm. The averaging time was set to 0.5 seconds and data interval to 0.333 nm, giving a scan rate of 40 nm/min (Figure 2). In the Options tab of the Setup dialog box, the slit width was set to 2 nm and both **Double beam mode** and **Baseline correction** were selected (Figures 3 and 4).

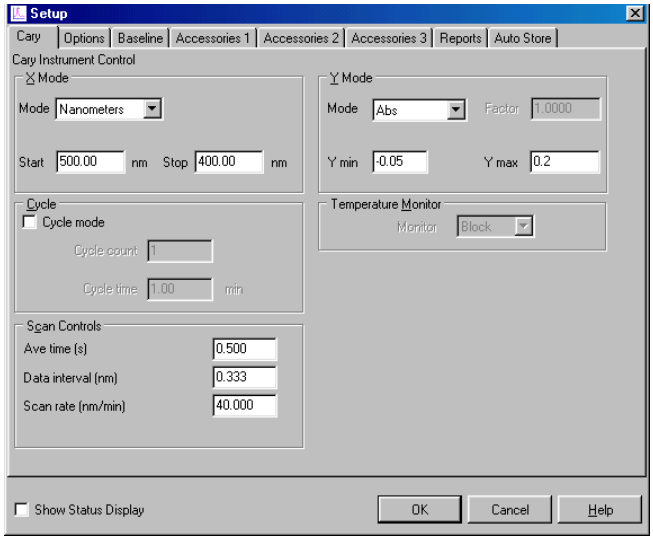

Figure 2. Cary 300 UV-Vis Spectrophotometer Setup dialog box settings

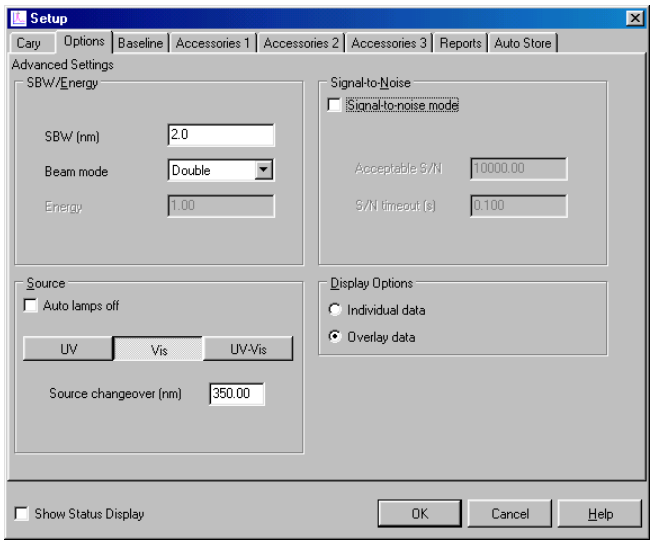

Figure 3. Cary 300 UV-Vis Spectrophotometer Options dialog box settings

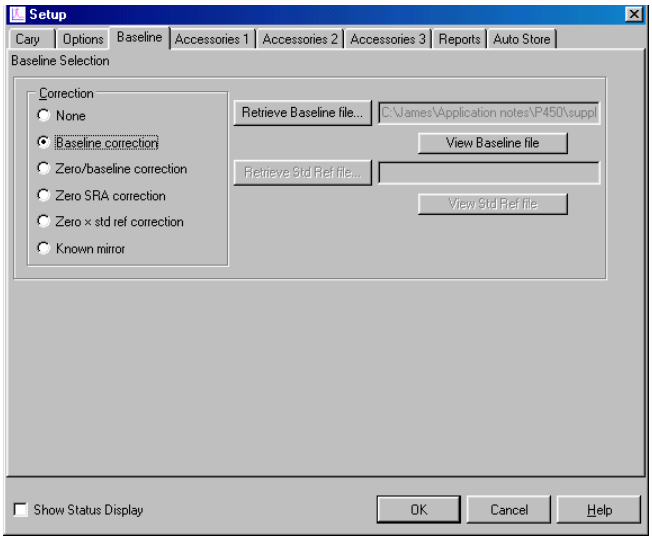

Figure 4. Cary 300 UV-Vis Spectrophotometer Baseline dialog box settings

Procedure for spectroscopic measurement:

1. Add a pinch of solid sodium dithionite to 6 mL concentrated cells or plasma membrane vesicles in PBG, and vortex well.

2. Wait two minutes to ensure complete reduction of cytochromes, vortex again, place 3 mL of sample into both the sample (S) and reference (R) cuvette and record the baseline.

3. Pipette S back into the glass conical-bottom tube and place the tube in a rack in the fume hood. Bubble S with CO at about 2 bubbles per second for 30 seconds. **Note: CO is a deadly poison. Use in fume hood only. DO NOT work alone.**

4. Record the S-R difference spectrum. A peak should be seen at lambda max = 448 nm.

Results and discussion

Cytochrome P-450 is a haemoprotein. When the haem Fe3+ is reduced to Fe2+ by the addition of sodium dithionite and complexed with carbon monoxide, a characteristic absorption spectrum can be measured. The reduced, carbon monoxide complexed, difference spectrum of cytochrome P-450, has a maximum absorbance at approximately 450 nm. The extinction coefficient, 91 mM, has been accurately determined for the wavelength couple 450 nm and 490 nm, making quantitative determination possible.

Initially, the cytochrome P-450 level in the DJ4309 bacterial culture was measured and a characteristic spectrum was observed (Figure 5). This bacterial strain expresses cytochrome P-450 at high levels and was used to confirm that the whole cell assay can effectively detect cytochrome P-450.

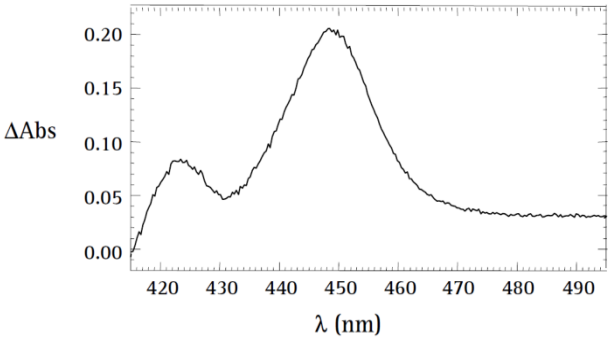

Figure 5. Baseline corrected scan of DJ4309 whole cells expressing high levels of cytochrome P-450

The sensitivity of the assay was tested using a bacterial strain that expresses cytochrome P-450 at much lower levels than DJ4309. In this case, the optical spectrum of both whole cells and plasma membrane vesicles was compared (Figure 3). The noise level was lower for the membrane preparation because the light scattering is reduced, but the shape of the whole-cell spectrum is nevertheless accurate. These results show that the whole cell assay can accurately detect cytochrome P-450 even when very low levels of the enzymes are present.

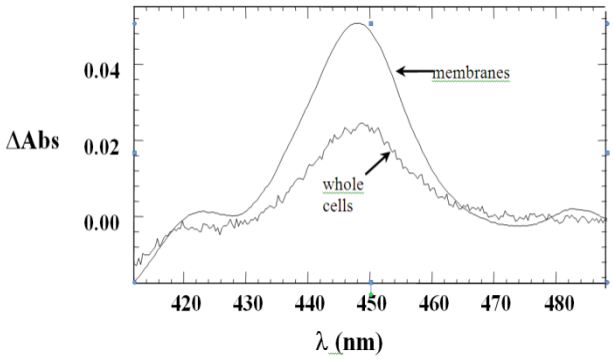

Figure 6. Baseline corrected scans of both whole cells and plasma membrane vesicles from a bacterial strain expressing low levels of cytochrome P-450

Cultures containing whole cells are highly turbid suspensions and have a high background optical density, making it difficult to detect very small changes in absorbance. This study has shown that using the Agilent Cary 300 UV-Vis allows these measurements to be taken directly on whole cells. For routine measurement of P450 expression, membrane vesicle preparation can therefore be avoided.

References

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9. Part Numbers:

Product	Part Number
Agilent Cary 300 UV-Vis Spectrophotometer	00 100691 00
10 mm pathlength rectangular quartz cuvette	66 100009 00
Cary WinUV Bio Package Software	85 101585 00

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