Vivid® CYP450 Screening Kits User Guide

Catalog no. P2856, P2857, P2858, P2859, P2860, P2861, P2862, P2863, P2864, P2969, P2970, P2972, P3019, P3021, PV6140, PV6141, and PV6142

Shipping: Varies **Storage:** Varies (see Kit Contents)

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Introduction

Vivid® CYP450 Screening Kits enable rapid measurement of interactions between drug candidates and cytochrome P450 enzymes using a simple "mix-and-read" fluorescent assay that is designed for high-throughput screening in multiwell plates. These kits will allow investigators to rapidly identify compound-CYP450 interactions, eliminating unsuitable compounds early in the drug discovery process. Vivid® CYP450 Screening Kits can also be used to generate predictive structure-activity relationship models to guide medicinal chemists in their design of compounds.

Test compounds are analyzed by their capacity to inhibit the production of a fluorescent signal in reactions using recombinant CYP450 isozymes and specific Vivid® Substrates. The availability of more than one structurally unrelated fluorogenic Vivid® Substrate for CYP2C9, CYP2D6, CYP3A4, and CYP3A5 reduces the potential for false negatives or false positives that could result from substrate-dependent interactions.

Kit Contents

Vivid® CYP450 Screening Kit	Contents	Part no.	Quantity	Storage
Vivid® CYP1A2 Blue (Cat. no. P2863)	Vivid® CYP450 Reaction Buffer I	P2881	50 mL	RT
	CYP1A2 BACULOSOMES® Plus Reagent	P2792	0.5 nmol	–80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	–20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	–20°C, light protected
Vivid® CYP2A6 Blue (Cat. no. PV6140)	Vivid® CYP450 Reaction Buffer II	P2913	50 mL	RT
	CYP2A6 BACULOSOMES® Plus Reagent	PV6137	0.5 nmol	–80°C
	Vivid® CC Substrate	PV6143	0.5 mg	–20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	–20°C, light protected
Vivid® CYP2B6 Blue (Cat. no. P3019)	Vivid® CYP450 Reaction Buffer I	P2881	50 mL	RT
	CYP2B6 BACULOSOMES® Plus Reagent	P3028	0.5 nmol	-80 °C
	Vivid® BOMCC Substrate	P2975	0.1 mg	–20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	–20°C, light protected
Vivid® CYP2C8 Green (Cat. no. PV6141)	Vivid® CYP450 Reaction Buffer II	P2913	50 mL	RT
	CYP2C8 BACULOSOMES® Plus Reagent	PV6138	0.5 nmol	–80°C
	Vivid® DBOMF Substrate	P2974	0.1 mg	–20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	–20°C, light protected
Vivid® CYP2C9 Blue (Cat. no. P2861)	Vivid® CYP450 Reaction Buffer II	P2913	50 mL	RT
	CYP2C9 BACULOSOMES® Plus Reagent	P2378	0.5 nmol	–80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	–20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	–20°C, light protected
Vivid® CYP2C9 Green (Cat. no. P2860)	Vivid® CYP450 Reaction Buffer II	P2913	50 mL	RT
	CYP2C9 BACULOSOMES® Plus Reagent	P2378	0.5 nmol	–80°C
	Vivid® BOMF Substrate	P2869	0.1 mg	–20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	–20°C, light protected
Vivid® CYP2C9 Red (Cat. no. P2859)	Vivid® CYP450 Reaction Buffer II	P2913	50 mL	RT
	CYP2C9 BACULOSOMES® Plus Reagent	P2378	0.5 nmol	–80°C
	Vivid® OOMR Substrate	P2868	0.1 mg	–20°C, light protected
	Vivid® Red Fluorescent Standard	P2874	0.1 µmol	–20°C, light protected
Vivid® CYP2C19 Blue (Cat. no. P2864)	Vivid® CYP450 Reaction Buffer II	P2913	50 mL	RT
	CYP2C19 BACULOSOMES® Plus Reagent	P2570	0.5 nmol	–80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	–20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	–20°C, light protected
Vivid® CYP2D6 Blue (Cat. no. P2972)	Vivid® CYP450 Reaction Buffer I	P2881	50 mL	RT
	CYP2D6 BACULOSOMES® Plus Reagent	P2283	0.5 nmol	–80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	–20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	–20°C, light protected

Vivid® CYP450 Screening Kit	Contents	Part no.	Quantity	Storage
Vivid® CYP2D6 Cyan (Cat. no. P2862)	Vivid® CYP450 Reaction Buffer I	P2881	50 mL	RT
	CYP2D6 BACULOSOMES® Plus Reagent	P2283	0.5 nmol	–80°C
	Vivid® MOBFC Substrate	P2871	0.1 mg	–20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	–20°C, light protected
Vivid® CYP2E1 Blue (Cat. no. P3021)	Vivid® CYP450 Reaction Buffer III	P2949	50 mL	RT
	CYP2E1 BACULOSOMES® Plus Reagent	P2948	1.0 nmol	–80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	–20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	–20°C, light protected
Vivid® CYP2J2 Cyan (Cat. no. PV6142)	Vivid® CYP450 Reaction Buffer II	P2913	50 mL	RT
	CYP2J2 BACULOSOMES® Plus Reagent	PV6139	0.5 nmol	–80°C
	Vivid® MOBFC Substrate	P2871	0.1 mg	–20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	–20°C, light protected
Vivid® CYP3A4 Blue (Cat. no. P2858)	Vivid® CYP450 Reaction Buffer I	P2881	50 mL	RT
	CYP3A4 BACULOSOMES® Plus Reagent	P2377	0.5 nmol	–80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	–20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	–20°C, light protected
Vivid® CYP3A4 Green (Cat. no. P2857)	Vivid® CYP450 Reaction Buffer I	P2881	50 mL	RT
	CYP3A4 BACULOSOMES® Plus Reagent	P2377	0.5 nmol	–80°C
	Vivid® DBOMF Substrate	P2974	0.1 mg	–20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	–20°C, light protected
Vivid® CYP3A4 Red (Cat. no. P2856)	Vivid® CYP450 Reaction Buffer I	P2881	50 mL	RT
	CYP3A4 BACULOSOMES® Plus Reagent	P2377	0.5 nmol	–80°C
	Vivid® BOMR Substrate	P2865	0.1 mg	–20°C, light protected
	Vivid® Red Fluorescent Standard	P2874	0.1 µmol	–20°C, light protected
Vivid® CYP3A5 Blue (Cat. no. P2970)	Vivid® CYP450 Reaction Buffer I	P2881	50 mL	RT
	CYP3A5 BACULOSOMES® Plus Reagent	P2512	0.5 nmol	–80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	–20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	–20°C, light protected
Vivid® CYP3A5 Green (Cat. no. P2969)	Vivid® CYP450 Reaction Buffer I	P2881	50 mL	RT
	CYP3A5 BACULOSOMES® Plus Reagent	P2512	0.5 nmol	–80°C
	Vivid® DBOMF Substrate	P2974	0.1 mg	–20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	–20°C, light protected

All kits also contain 0.5 mL Vivid® Regeneration System, 100X (Part no. P2878; 333 mM Glucose-6-phosphate and 30 U/mL Glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate, pH 8.0) and 0.5 mL Vivid® $NADP^+$ (Part no. P2879; 10 mM $NADP^+$ in 100 mM potassium phosphate, pH 8.0). Store both components at –80°C.

- The Vivid® CYP450 Reaction Buffers are 200 mM (Reaction buffer I), 100 mM (Reaction buffer II), or 400 mM (Reaction buffer III) potassium phosphate pH 8.0.
- CYP450 BACULOSOMES® Plus Reagents consist of recombinant human cytochrome P450, human cytochrome P450 reductase, and in some cases, human cytochrome b_5.
- The Vivid® Substrates and Standards are supplied as a dried film. Reconstitution is necessary before use.

Materials Required but not Supplied

- Multi-well black plates suitable for fluorescence measurements. We recommend using 96-well black, non-treated polystyrene assay plates (Corning/Costar, Cat. no. 3915).
- Fluorescence plate reader with filters as described in Table 7 (page 11)
- Pipetting devices
- Reagent reservoirs
- Acetonitrile, anhydrous
- DMSO
- Nanopure water
- P450 inhibitor (see Suggested Inhibitors, page 12) for use as a control inhibitor

Optional Materials not Supplied

- 0.5 M Tris base for use as a Stop Reagent. This solution can be prepared by dissolving 60.6 g of Tris base (e.g., Sigma-Aldrich, Cat. no. T1503) in 1 L of nanopure water. The pH of 0.5 M Tris base will be approximately 10.8. Note that the Stop Reagent should be Tris base and not Tris-HCl. Store at room temperature.

Storage and Stability

- Vivid® Substrates and Fluorescent Standards are stable for at least six months when stored desiccated and protected from light at –20°C.
- For long-term storage, reconstituted Vivid® Substrates and Fluorescent Standards should be kept desiccated at –20°C. DMSO solutions are hygroscopic, and cold vials should be warmed to ambient temperature before opening. After opening, they should be capped promptly to avoid reagent dilution by absorbed moisture.
- The CYP450 BACULOSOMES® Plus Reagent should be stored at –80°C.
- The Vivid® Regeneration System should be stored at –80°C. Upon first thaw, aliquot into single use vials as the reagent should not be subjected to additional freeze/thaw cycles.
- The Vivid® $NADP^+$ should be stored at –80°C and is stable for at least 10 freeze/thaw cycles. Store protected from light.
- The Vivid® CYP450 Reaction Buffer (2X) should be stored at room temperature.

Assay Theory

Vivid® CYP450 Screening Kits are designed to assess metabolism and inhibition of human P450 isozymes involved in hepatic drug metabolism: CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, and CYP3A5. The kits employ Vivid® Substrates and CYP450 BACULOSOMES® Plus Reagents. The CYP450 BACULOSOMES® Plus Reagents are microsomes prepared from insect cells expressing a human P450 isozyme, human cytochrome P450 reductase, and in some cases, human cytochrome b_5. CYP450 BACULOSOMES® Plus Reagents offer a distinct advantage over human liver microsomes in that only one CYP450 enzyme is expressed, thereby preventing metabolism by other CYP450s. The Vivid® Substrates are metabolized by a specific CYP450 enzyme into products that are highly fluorescent in aqueous solutions. Figure 1 schematically depicts the metabolism of a Vivid® Substrate into a fluorescent metabolite. Note that the Vivid® Substrates have one or two potential sites for metabolism (indicated by arrows in Figure 1) and that oxidation at either site releases the highly fluorescent metabolite. For structures of the Vivid® Substrates and Standards, visit us online at **www.lifetechnologies.com/vivid**.

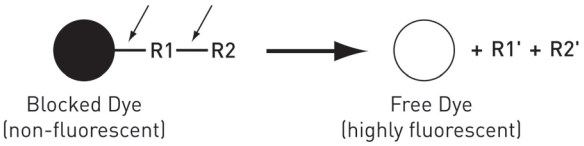

Figure 1 Schematic of the metabolism of the "blocked" dye substrate into a fluorescent metabolite

The fluorescent metabolites are excited in the visible light spectrum, which minimizes interference caused by the background fluorescence of UV-excitable compounds and NADPH. The excellent reaction kinetics and optical properties of the Vivid® Substrates allow their use at concentrations at or below their K_m value in a reaction with P450 isozymes, assuring detection of even weak inhibitors and providing the convenience of room temperature or 37°C incubations. The Vivid® CYP450 Assay may be run in a kinetic or endpoint mode (which is illustrated in Figure 2).

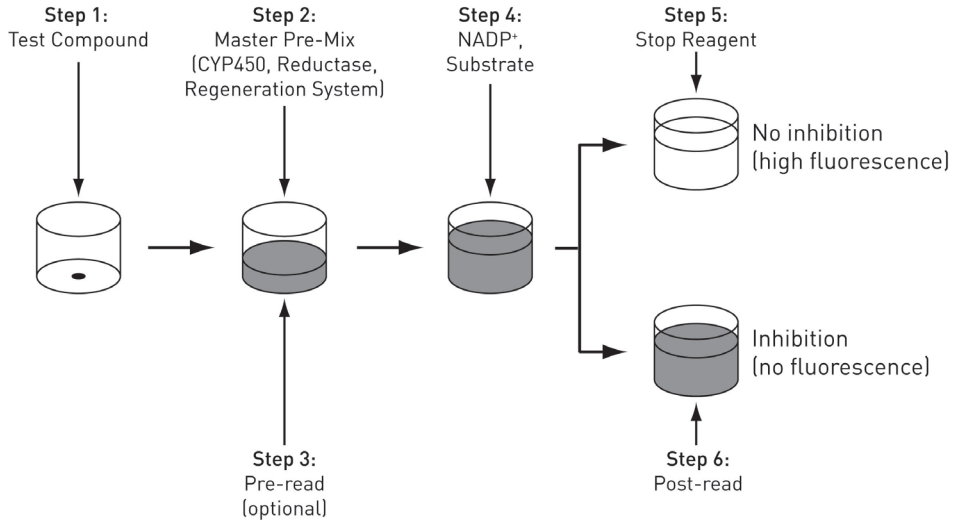

Figure 2 A schematic representation of an endpoint Vivid® CYP450 Assay

In end point mode, the test compounds (Step 1) are first combined with the Master Pre-mix (Step 2), consisting of CYP450 BACULOSOMES® Plus Reagents and the Regeneration System (consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase). The Regeneration System converts $NADP^+$ into NADPH, which is required to start the CYP450 reaction. After a brief pre-incubation, the background fluorescence of the test compound and Master Pre-mix is measured (Step 3, Pre-read). The enzymatic reaction is initiated by the addition of a mix of $NADP^+$ and the appropriate Vivid® Substrate (Step 4) and plate is incubated for the desired reaction time. After the addition of a Stop Reagent (Step 5), the fluorescence is measured (Step 6).

In kinetic mode, the fluorescence is measured continuously starting after Step 4 (and eliminating Steps 5 and 6). Standard curves, constructed from the supplied Fluorescent Standard, can be used to calculate reaction rates from the observed fluorescence intensities in both assay formats. Assay parameters for all isoforms are listed in Tables 4 and 5 (pages 8 and 9, respectively).

Vivid® CYP450 High-Throughput Screening Assay Protocol

Each complete reaction must contain CYP450 BACULOSOMES® Plus Reagent, Vivid® Substrate, Vivid® NADP+, and Vivid® Regeneration System in the appropriate Vivid® CYP450 Reaction Buffer. There are two possible modes for this assay: kinetic and endpoint. The method you choose will depend on your analytical needs and the equipment available.

- The kinetic mode is useful for analysis of one multi-well plate at a time and does not require the addition of the stop reagent.
- In endpoint mode, after an appropriate incubation time, the reaction is stopped by the addition of stop solution. Endpoint mode allows the reaction to be performed in several multi-well plates simultaneously.

Note: The following protocol is configured for use with one 96-well plate and 100 µL reactions. However, the protocol can be modified to accommodate several different plate formats by adjusting the calculations for the number of wells (and volume per well) in your experiment. Each kit supplies enough reagents for at least 300 × 100 µL reactions.

Suggested assay conditions for screening with Vivid® kits are described in Table 1, below.

Table 1 Assay conditions

Condition	Purpose	Dispensing
Test Compound	Screen for inhibition by compound of interest	40 µL 2.5X test compound 50 µL Master Pre-Mix 10 µL Vivid® Substrate and NADP+
Positive Inhibition Control	Inhibits the reaction with a known P450 inhibitor and enables the subtraction of background during data analysis	40 µL 2.5X positive inhibition control (see page 12) 50 µL Master Pre-Mix 10 µL Vivid® Substrate and NADP+
Solvent Control (No inhibitor)	Accounts for possible solvent inhibition caused by introduction of test compounds originally dissolved in an organic solvent such as DMSO	40 µL 2.5X solvent control 50 µL Master Pre-Mix 10 µL Vivid® Substrate and NADP+

Thaw Reagents

1. Place the CYP450 BACULOSOMES® Plus Reagent, Vivid® Regeneration System, and Vivid® NADP+ at room temperature just until thawed (10–15 minutes), then store on ice until ready to use. Mix the CYP450 BACULOSOMES® Plus Reagent and Vivid® Regeneration System gently by inversion to obtain a homogenous mixture after thawing. Do **not** vortex CYP450 BACULOSOMES® Plus Reagent or Vivid® Regeneration System.
2. Allow the Vivid® Substrate and Fluorescent Standard to warm to ambient room temperature (10–15 minutes) prior to reconstitution.

Reconstitute Vivid® Substrate and Fluorescent Standard

1. Reconstitute the Vivid® Substrate using anhydrous acetonitrile and the Fluorescent Standard using DMSO (see Tables 2 and 3, below).
2. Keep these solutions at room temperature for immediate use (≤ 4 hours), or store at -20°C.

Table 2 Reconstitution of the Vivid® Substrates

P450	Vivid® Substrate	Molecular weight	mg per tube	µmol per tube	µL acetonitrile added per tube	[stock solution] (mM)	[screening concentration] (µM)
1A2	Vivid® EOMCC	245.2	0.1	0.41	205	2	3
2A6	Vivid® CC	171.2	0.5	2.92	290	10	10
2B6	Vivid® BOMCC	307.3	0.1	0.32	160	2	3
2C8	Vivid® DBOMF	572.6	0.1	0.17	85	2	1
2C9	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMF	452.5	0.1	0.22	110	2	1
	Vivid® OOMR*	355.4	0.1	0.28	140	2	1
2C19	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
2D6	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
	Vivid® MOBFC	350.3	0.1	0.28	140	2	5
2E1	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
2J2	Vivid® MOBFC	350.3	0.1	0.28	140	2	3
3A4	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® DBOMF	572.6	0.1	0.17	85	2	2
	Vivid® BOMR	333.3	0.1	0.30	150	2	3
3A5	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® DBOMF	572.6	0.1	0.17	85	2	2

*Heat Vivid® OOMR Substrate at 50°C for 3–5 minutes and vortex to reconstitute.

Table 3 Reconstitution of the Fluorescent Standard. Use the blank cells in the table for your calculations. The value X is the amount of Standard listed on the tube label.

Assay Standard	µmol per tube (X)	Reconstitution Solvent	µL Reconstitution Solvent added per tube (X x 10000)	[Fluorescent Standard] after reconstitution, µM
Example	**0.1**	**DMSO**	**1000 µL**	**100**
Blue Standard		DMSO		100
Cyan Standard		DMSO		100
Green Standard		DMSO		100
Red Standard		DMSO/water (1:1)		100

Prepare 1X Vivid® CYP450 Reaction Buffer

1. Dilute 2X Vivid® CYP450 Reaction Buffer with nanopure water according to the Table 4, below. This typically provides enough 1X buffer for one 96-well plate; however, you can scale up or down the volumes as needed. Use the diluted buffer for the preparation of standards, inhibitors, Master Pre-Mix, and Vivid® Substrate/NADP+ solutions. Store the 1X buffer at room temperature.

Note: The CYP2A6 assay requires Vivid® CYP450 Reaction Buffer II at 0.5X as its final concentration. All other isoforms require the indicated buffer at 1X as the final concentration.

Table 4 Vivid® CYP450 Reaction Buffer Preparation

P450	Vivid® CYP450 Reaction Buffer	Concentration before dilution	Desired concentration after dilution	Volume of buffer	Volume of water
1A2	Buffer I	200 mM	100 mM	10 mL	10 mL
2A6	Buffer II	100 mM	25 mM	5 mL	15 mL
2B6	Buffer I	200 mM	100 mM	10 mL	10 mL
2C8	Buffer II	100 mM	50 mM	10 mL	10 mL
2C9	Buffer II	100 mM	50 mM	10 mL	10 mL
2C19	Buffer II	100 mM	50 mM	10 mL	10 mL
2D6	Buffer I	200 mM	100 mM	10 mL	10 mL
2E1	Buffer III	400 mM	200 mM	10 mL	10 mL
2J2	Buffer II	100 mM	50 mM	10 mL	10 mL
3A4	Buffer I	200 mM	100 mM	10 mL	10 mL
3A5	Buffer I	200 mM	100 mM	10 mL	10 mL

Prepare Standard Curve (Optional)

We recommend that you use at least seven points (in addition to the blank) for the standard curve and perform it in duplicate. The sample method below describes a seven-point standard curve set up in columns 1 and 2 of the 96-well assay plate.

1. Dilute the Vivid® Fluorescent Standard to 500 nM by mixing 5 µL of 100 µM Standard (Table 3, page 7) with 995 µL of 1X Vivid® CYP450 Reaction Buffer.
2. Add 200 µL of 500 nM Vivid® Fluorescent Standard to wells A1 and A2.
3. Add 100 µL of 1X Vivid® CYP450 Reaction Buffer to each of the remaining wells in columns 1 and 2.
4. Transfer 100 µL from wells A1 and A2 into the wells below (B1 and B2, respectively) containing 100 µL of 1X Vivid® CYP450 Reaction Buffer and mix by pipetting. This is a two-fold dilution.
5. Repeat this dilution step, leaving wells H1 and H2 as an assay blank containing only 1X Vivid® CYP450 Reaction Buffer and no Standard. The resulting Fluorescent Standard concentrations are: 500 nM, 250 nM, 125 nM, 62.5 nM, 31.25 nM, 15.625 nM, 7.8125 nM, and 0 nM.

Note: These are suggested initial concentrations for the standard curve. More or less may be appropriate depending on your experimental needs.

Note: The assay can be performed simply by using fluorescence values instead of converting to concentration of product formed.

Prepare Test Compounds, Positive Inhibition Control, and Solvent Control

1. Prepare 2.5X Test Compounds by dilution into 1X Vivid® CYP450 Reaction Buffer.
2. Prepare a 2.5X solution of a known P450 Inhibitor in 1X Vivid® CYP450 Reaction Buffer for positive control of inhibition.

Note: We recommend use of the inhibitors listed in Table 8, page 12.

3. Prepare a solution of the solvent used to dissolve the test compounds and known P450 inhibitor at 2.5X final concentration in 1X Vivid® CYP450 Reaction Buffer.

Note: P450 activity can be greatly affected by organic solvents commonly used to dissolve test compounds. For example solvent inhibition data, visit us online at **www.lifetechnologies.com/vivid**.

Dispense Test Compounds, Positive Inhibition Control, and Solvent Control

1. Add 40 µL of the 2.5X solutions prepared above to desired wells of the plate.
2. We recommend at least three replicates for the Positive Inhibition Control and Solvent Control.

Prepare and Dispense Master Pre-Mix

1. Prepare the Master Pre-Mix by diluting P450 BACULOSOMES® Plus Reagent and Vivid® Regeneration System in 1X Vivid® CYP450 Reaction Buffer (see Table 5, below). Mix by inversion. Store this solution at room temperature for immediate use.

Note: Table 5, below, describes the preparation of Master Pre-mix for one entire 96-well plate. Depending on your experimental needs, you may prepare more or less Master Pre-mix.

2. Dispense 50 µL of Master Pre-Mix to each well.

Table 5 Master Pre-mix (mixture of CYP450 BACULOSOMES® Plus Reagent and Vivid® Regeneration System)

P450	Vivid® Substrate	µL of 1X Vivid® CYP450 Reaction Buffer added	µL of Vivid® Regeneration System (100X) added	µL of CYP450 BACULOSOMES® Plus added	Concentration of P450 in Master Pre-mix (2X), nM	Screening concentration of P450, nM*
1A2	Vivid® EOMCC	4850 (Buffer I)	100	50	10	5
2A6	Vivid® CC	4800 (0.5X Buffer II)	100	100	20	10
2B6	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
2C8	Vivid® DBOMF	4850 (Buffer II)	100	50	10	5
2C9	Vivid® BOMCC	4800 (Buffer II)	100	100	20	10
	Vivid® BOMF	4800 (Buffer II)	100	100	20	10
	Vivid® OOMR	4800 (Buffer II)	100	100	20	10
2C19	Vivid® EOMCC	4850 (Buffer II)	100	50	10	5
2D6	Vivid® EOMCC	4800 (Buffer I)	100	100	20	10
	Vivid® MOBFC	4750 (Buffer I)	100	150	30	15
2E1	Vivid® EOMCC	4700 (Buffer III)	100	100	40	20
2J2	Vivid® MOBFC	4850 (Buffer II)	100	50	10	5
3A4	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
	Vivid® DBOMF	4850 (Buffer I)	100	50	10	5
	Vivid® BOMR	4850 (Buffer I)	100	50	10	5
3A5	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
	Vivid® DBOMF	4850 (Buffer I)	100	50	10	5

*For your first experiment, we suggest these concentrations of P450. Based on your results, you may find more or less enzyme is necessary.

Pre-Incubate

1 Incubate the plate for 10 minutes at room temperature to allow the compounds to interact with the P450 in the absence of enzyme turnover.

2. During this pre-incubation, prepare a 10X mixture of Vivid® Substrate and Vivid® NADP+ (Table 6, below).

Note: Table 6, below, describes the preparation of a Vivid® Substrate and Vivid® NADP+ mixture for one entire 96-well plate. Depending on your experimental needs, you may prepare more or less of this reagent.

3. You may also wish to include a pre-read at this point to determine if your compounds are fluorescent.

Table 6 Vivid® Substrate and Vivid® NADP+ Mixture

P450	Vivid® Substrate	µL of 1X Vivid® CYP450 Reaction Buffer added	µL of Reconstituted Substrate added*	µL of Vivid® NADP+ (100X) added	[Vivid® Substrate] in 10X mixture (µM)	[Vivid® NADP+] in 10X mixture (µM)
1A2	Vivid® EOMCC	955 (Buffer I)	15	30	30	300
2A6	Vivid® CC	960 (0.5X Buffer II)	10	30	100	300
2B6	Vivid® BOMCC	955 (Buffer I)	15	30	30	300
2C8	Vivid® DBOMF	895 (Buffer II)	5	100	10	1000
2C9	Vivid® BOMCC	920 (Buffer II)	50	30	100	300
	Vivid® BOMF	895 (Buffer II)	5	100	10	1000
	Vivid® OOMR	895 (Buffer II)	5	100	10	1000
2C19	Vivid® EOMCC	920 (Buffer II)	50	30	100	300
2D6	Vivid® EOMCC	920 (Buffer I)	50	30	100	300
	Vivid® MOBFC	945 (Buffer I)	25	30	50	300
2E1	Vivid® EOMCC	920 (Buffer III)	50	30	100	300
2J2	Vivid® MOBFC	955 (Buffer II)	15	30	30	300
3A4	Vivid® BOMCC	920 (Buffer I)	50	30	100	300
	Vivid® DBOMF	890 (Buffer I)	10	100	20	1000
	Vivid® BOMR	885 (Buffer I)	15	100	30	1000
3A5	Vivid® BOMCC	920 (Buffer I)	50	30	100	300
	Vivid® DBOMF	890 (Buffer I)	10	100	20	1000

*See Reconstitute Vivid® Substrate and Fluorescent Standard, page 7.

Start Reaction

1. Start the reaction by adding 10 µL per well of the 10X Vivid® Substrate and NADP+ mixture prepared in Pre-Incubation step (Table 6, page 10).

Measure Fluorescence

1. **Kinetic Assay Mode (recommended):** Immediately (less than 2 minutes) transfer the plate into the fluorescent plate reader and monitor fluorescence over time at excitation and emission wavelengths listed in Table 7, page 11. Initially, we recommend reads in 1-minute intervals for 60 minutes. Based on these initial results, you can further refine the measurement times for subsequent assays, as desired. Appropriate reaction times will vary by kit and experimental conditions.

2. **Endpoint Assay Mode:** Incubate the plate for the desired amount of time, and then add 50 µL of Stop Reagent (0.5 M Tris base) to each well to quench the reaction. Measure fluorescence in the fluorescent plate reader at excitation and emission wavelengths listed in Table 7, page 11.

3. Proceed to data analysis, page 11.

Table 7 Recommended excitation and emission wavelengths and filter sets

		Vivid® Fluorescent Standard*							
		Red		Blue		Green		Cyan	
Fluorescence Plate Reader	Excitation/ Emission	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width
monochromator	excitation	550	12	415	20	490	12	415	20
	emission	590	12	460	20	520	12	520	20
filter-based	excitation	535	25	415	20	485	20	415	20
	emission	590	20	460	20	520	25	520	25

*Red Standard is sodium salt of resorufin. Green Standard is fluorescein. Blue Standard is 3-cyano-7-hydroxycoumarin. Cyan Standard is 7-hydroxy-4-trifluoromethylcoumarin. We recommend exciting the Cyan and Blue Standards off-peak (>400 nm) to minimize background from NADPH fluorescence.

Suggested Protocol for the Analysis of Results

Kinetic Assay Mode

1. Obtain reaction rates by calculating the change in fluorescence per unit time.
2. Calculate the percent inhibition due to presence of test compound or positive inhibition control using the equation:

$$\%\ \text{Inhibition} = \left(1 - \frac{X - B}{A - B}\right) \times 100\%$$

where X is the rate observed in the presence of test compound, A is the rate observed in the absence of inhibitor (solvent control or no inhibitor control, as appropriate), and B is the rate observed in the presence of the positive inhibition control.

Endpoint Assay Mode

1. Calculate percent inhibition due to presence of test compound or positive inhibition using the following equation:

$$\%\ \text{Inhibition} = \left(1 - \frac{X - B}{A - B}\right) \times 100\%$$

where X is the fluorescence intensity observed in the presence of test compound, A is the fluorescence intensity observed in the absence of inhibitor (solvent control or no inhibitor control, as appropriate), and B is the fluorescence intensity observed in the presence of the positive inhibition control.

Optional: Both types of data analysis above can be performed using a standard curve (see Prepare Standard Curve, page 8) to calculate the reaction rates as nmol product formed per unit time, if desired.

Suggested Inhibitors

Table 8 Suggested Inhibitors

Enzyme	Inhibitor	Recommended Vendor (Cat. no.)	Suggested Final Concentration (1X)
CYP1A2	α-naphthoflavone	Sigma-Aldrich (N5757)	10 µM
CYP2A6	tranylcypromine	Sigma-Aldrich (P8511)	100 µM
CYP2B6	miconazole	Sigma-Aldrich (M3512)	30 µM
CYP2C8	montelukast	Cayman Chemical (10008318)	10 µM
CYP2C9	sulfaphenazole	Sigma-Aldrich (S0758)	30 µM
CYP2C19	miconazole	Sigma-Aldrich (M3512)	30 µM
CYP2D6	quinidine	Sigma-Aldrich (Q3625)	10 µM
CYP2E1	tranylcypromine	Sigma-Aldrich (P8511)	1 mM
CYP2J2	terfenadine	Sigma-Aldrich (T9652)	25 µM
CYP3A4	ketoconazole	Sigma-Aldrich (K1003)	10 µM
CYP3A5	ketoconazole	Sigma-Aldrich (K1003)	10 µM

References

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Trubetskoy, O.V., *et al.* (2005) Highly miniaturized formats for in vitro drug metabolism assays using Vivid® fluorescent substrates and recombinant human cytochrome P450 enzymes. *J. Biomol. Screen.* **10:**56-66.

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