Vivid® CYP450 Screening Kits Protocol

Cat. no. P2856, P2857, P2858, P2859, P2860, P2861, P2862, P2863, P2864, P2968, P2969, P2970, P2971, P2972, P3019, P3020 and P3021

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TABLE OF CONTENTS

1.0 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® CYP450 Substrates. The availability of more than one structurally unrelated fluorogenic Vivid® CYP450 Substrate for CYP3A4, CYP3A5, CYP2C9, CYP2B6 and CYP2D6 reduces the potential for false negatives (and false positives) that could result from substrate-dependent interactions.

2.0 MATERIALS SUPPLIED

Vivid® CYP450 Screening Kit	Description	Cat. no.	Quantity	Storage
Vivid® CYP1A2 Blue (P2863)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP1A2 BACULOSOMES® 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® CYP2B6 Blue (P3019)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2B6 BACULOSOMES® 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® CYP2B6 Cyan (P3020)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2B6 BACULOSOMES® Reagent	P3028	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Blue (P2861)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® 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 (P2860)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® 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 (P2859)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® 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 (P2864)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C19 BACULOSOMES® 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 (P2972)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2D6 BACULOSOMES® Reagent	P2283	0.5 nmol x 2	-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 Cyan (P2862)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2D6 BACULOSOMES® Reagent	P2283	0.5 nmol x 2	-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 (P3021)	Vivid® CYP450 Reaction Buffer III	P2949	50 ml	RT
	CYP2E1 BACULOSOMES® 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® CYP3A4 Blue (P2858)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® 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 Cyan (P2968)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Green (P2857)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® 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 (P2856)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® 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 (P2970)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® 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 Cyan (P2971)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Green (P2969)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® 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 Regeneration System, 100X (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 NADP+ (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® Reagents consist of recombinant human Cytochrome P450 (1 µM) and rabbit NADPH P450 Reductase.
- The Vivid® Substrates and Standards are supplied as a dried film. Reconstitution is necessary before use.

2.1 Materials Required but not Supplied

- Multiwell black plates suitable for fluorescence measurements (Note: black-walled, clear bottom plates are needed for bottom-read fluorescent microplate readers). Invitrogen recommends using Costar #3915 non-treated plates
- Fluorescence plate reader with filters as described in Table 6
- Pipeting devices
- Reagent reservoir(s)
- Acetonitrile, anhydrous
- DMSO, reagent grade
- Deionized water
- Stop Reagent (CYP450 isozyme specific inhibitor) if performing an endpoint assay or in kinetic mode for the positive control of inhibition. For more information on inhibitors, see Section 7.0.

3.0 STORAGE AND STABILITY

Vivid® CYP450 Substrates and Fluorescent Standards are stable for at least six months when stored desiccated and protected from light at –20°C. For short-term storage, acetonitrile- or DMSO-based stock solutions should be stored in a desiccator at 4°C. Long-term storage requires that organic solutions 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® Reagent should be stored at -80°C. No significant decrease in activity (see enclosed Certificate of Analysis) was observed after 5 freeze/thaw cycles except for CYP2D6 which showed a 5% decrease. The 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 $NADP^+$ 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) can be stored at 4°C or room temperature.

4.0 ASSAY THEORY

Vivid® CYP450 Screening Kits are designed to assess metabolism and inhibition of the predominant human P450 isozymes involved in hepatic drug metabolism: CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5. The kits employ Vivid® CYP450 Substrates and CYP450 BACULOSOMES® Reagents. The CYP450 BACULOSOMES® Reagents are microsomes prepared from insect cells expressing a human P450 isozyme and rabbit NADPH-P450 reductase (CYP2E1 also contains human cytochrome b_5). CYP450 BACULOSOMES® 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® CYP450 Substrate into a fluorescent metabolite. Note that the Vivid® Substrates have two potential sites for metabolism (indicated by arrows in Figure 1) and that oxidation at either site releases the highly fluorescent metabolite.

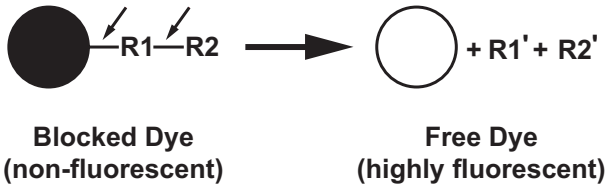

Figure 1. Schematic of the metabolism of the "blocked" dye substrate into a fluorescent metabolite

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 CYP450 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).

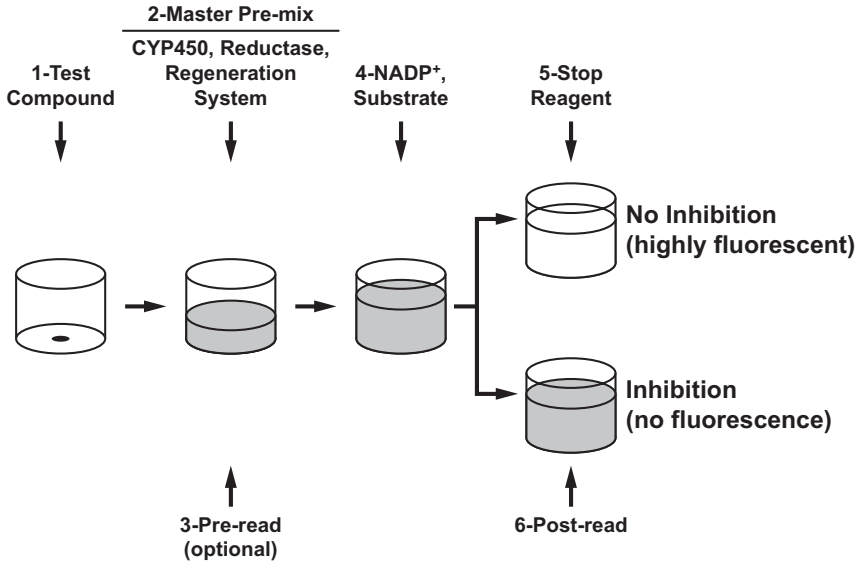

Figure 2. A schematic representation of an endpoint Vivid® CYP450 Assay

In end point (Section 5.1.9.2) mode, the test compounds (Step 1) are first combined with the Master Pre-mix (Step 2), consisting of CYP450 BACULOSOMES® 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 in Step 6.

In kinetic mode (Section 5.1.9.1), 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 isozymes CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 are listed in Tables 4 and 5.

5.0 VIVID® CYP450 HIGH-THROUGHPUT SCREENING ASSAY PROTOCOL

Each complete reaction must contain CYP450 BACULOSOMES® Reagent, Vivid® CYP450 Substrate, NADP+ and Regeneration System, all in the appropriate Vivid® CYP450 Reaction Buffer (supplied with each kit as a 2X solution). 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 multiwell 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 the CYP450 isozyme-specific inhibitor. Running in endpoint mode allows the reaction to be performed in several multiwell 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. See Trubetsoy *et al.* (2005) (see Section 9.0 for a complete list of references) for use of Vivid® kits in 1536-well plate formats. Each kit supplies enough reagents for at least 300 x 100 µl reactions.

5.1 Assay Procedure

5.1.1 Thaw Reagents

1. Thaw the P450 BACULOSOMES®, Regeneration System, and NADP+ on ice until ready to use. Do not vortex P450 BACULOSOMES® or Regeneration System.
2. Suggested assay conditions for screening with Vivid® kits are described in Table 1.

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	Inhibit the reaction with a known P450 inhibitor	40 µl 2.5X positive inhibition control (see Section 7.0) 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+
Background	Enables subtraction of background fluorescence during data analysis	40 µl 2.5X solvent control 50 µl Vivid® CYP450 Reaction Buffer 10 µl Vivid Substrate and NADP+

5.1.2 Reconstitution of Vivid® Substrate and Fluorescent Standard

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

Table 2. Reconstitution of the Vivid® CYP450 substrates

Isozyme Type	Vivid® CYP450 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
2B6	Vivid® BOMCC	307.3	0.1	0.32	160	2	5
	Vivid® BOMFC	350.3	0.1	0.28	140	2	2
2C9	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMF	452.5	0.1	0.22	110	2	2
	Vivid® OOMR§	355.4	0.1	0.28	140	2	2
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
3A4	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMFC	350.3	0.1	0.28	140	2	5
	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® BOMFC	350.3	0.1	0.28	140	2	5
	Vivid® DBOMF	572.6	0.1	0.17	85	2	2

§ Heat at 70°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 Assay 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	**0.11**	**DMSO**	**1100 µl**	**100**
Red Standard		DMSO/water (1:1)		100
Green Standard		DMSO		100
Blue Standard		DMSO		100
Cyan Standard		DMSO		100

5.1.3 Prepare Standard Curve (Optional)

1. With room temperature water, dilute enough Reaction Buffer (2X) to prepare enough 1X Reaction Buffer for your standard curve. In a 96-well plate, one standard curve can be run in 8 wells using 1 ml of Reaction Buffer. We recommend that at least six points (in addition to the blank) be used for the standard curve and that it be performed in duplicate.
2. To the first well of the column add 195 µl 1X Reaction Buffer.
3. Add 100 µl of 1X Reaction Buffer to each of the remaining wells in the column.
4. Add 5 µl of Fluorescent Standard (Table 3) to the first well containing 195 µl of buffer to achieve a starting concentration of 2.5 µM. Mix well.
5. Transfer 100 µl from this well into the next well containing 100 µl 1X Reaction Buffer and mix by pipetting. This is a two-fold dilution.
6. Repeat this dilution step, leaving the last well as an assay blank containing 1X Reaction Buffer only and no standard. The resulting Fluorescent Standard concentrations are: 2.5 µM, 1.25 µM, 625 nM, 312.5 nM, 156.25 nM, 78.125 nM, 39.063 nM and 0 nM.

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

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

5.1.4 Prepare Test Compounds, Positive Inhibition Control, and Solvent Control

1. Prepare 2.5X Test Compounds by dilution into deionized water. (For IC_{50} determination, a serial dilution of the test compound is required.)
2. Prepare a 2.5X solution of a known P450 Inhibitor in deionized water for positive control of inhibition (optional).

   Note: We recommend use of the inhibitors listed in Section 7.0.
3. Prepare a solution of the solvent used to dissolve the test compounds and known P450 inhibitor at 2.5X final concentration.

   Note: See Section 8.0 for information about particular solvents and tolerances.

5.1.5 Dispense Test Compounds, Positive Inhibition Control, and Solvent Control

1. Add 40 µl of the 2.5X solutions prepared in Section 5.1.4 to desired wells of the plate.
2. We recommend at least three replicates for the Positive Inhibition Control and Solvent Control.

5.1.6 Prepare and Dispense Master Pre-Mix

1. Prepare the Master Pre-Mix by diluting P450 BACULOSOMES® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer (2X) on ice (see Table 4). Mix by inversion.
2. Dispense 50 µl of Master Pre-Mix to each well. Mix.

   Note: To account for background fluorescence in the absence of CYP450 activity, dispense 50 µl of Vivid® CYP450 Reaction Buffer without P450 BACULOSOMES® to desired wells of the plate.

Table 4. Master Pre-mix (pre-mix of CYP450 BACULOSOMES® Reagents and Regeneration System). Keep on ice until ready to use

Isozyme Type	Vivid® CYP450 Substrate	µl of Vivid® CYP450 Reaction Buffer (2X) added	µl of Regeneration System (100X) added	µl of CYP450 BACULOSOMES® added	Concentration of CYP450 in Master Pre-mix (2X), nM	Screening concentration of CYP450, nM¶
1A2	Vivid® EOMCC	4850 (Buffer I)	100	50	10	5
2B6	Vivid® BOMCC	4800 (Buffer I)	100	100	20	10
	Vivid® BOMFC	4880 (Buffer I)	100	20	4	2
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	4700 (Buffer I)	100	200	40	20
2E1	Vivid® EOMCC	4850 (Buffer III)	100	50	10	5
3A4	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
	Vivid® BOMFC	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® BOMFC	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 the CYP450 enzyme. Based on your results, you may find more or less enzyme is necessary.

5.1.7 Pre-Incubate

1 Incubate the plate for 20 minutes at room temperature to allow the compounds to interact with the CYP450 in the absence of enzyme turnover.

2. During this pre-incubation, prepare the pre-mixture of Vivid® Substrate and $NADP^+$ (see Table 5).

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

Isozyme Type	Vivid® CYP450 Substrate	µl of Vivid® CYP450 Reaction Buffer (2X) added	µl of Reconstituted Substrate added (Section 5.1.2)	µl of $NADP^+$ (100X) added	Final % ACN from substrate
1A2	Vivid® EOMCC	885 (Buffer I)	15	100	0.15
2B6	Vivid® BOMCC	875 (Buffer I)	25	100	0.25
	Vivid® BOMFC	960 (Buffer I)	10	30	0.10
2C9	Vivid® BOMCC	850 (Buffer II)	50	100	0.50
	Vivid® BOMF	890 (Buffer II)	10	100	0.10
	Vivid® OOMR	890 (Buffer II)	10	100	0.10
2C19	Vivid® EOMCC	850 (Buffer II)	50	100	0.50
2D6	Vivid® EOMCC	850 (Buffer I)	50	100	0.50
	Vivid® MOBFC	945 (Buffer I)	25	30	0.25
2E1	Vivid® EOMCC	850 (Buffer III)	50	100	0.50
3A4	Vivid® BOMCC	850 (Buffer I)	50	100	0.50
	Vivid® BOMFC	945 (Buffer I)	25	30	0.25
	Vivid® DBOMF	890 (Buffer I)	10	100	0.10
	Vivid® BOMR	885 (Buffer I)	15	100	0.15
3A5	Vivid® BOMCC	850 (Buffer I)	50	100	0.50
	Vivid® BOMFC	945 (Buffer I)	25	30	0.25
	Vivid® DBOMF	890 (Buffer I)	10	100	0.10

5.1.8 Start Reaction

1. Start the reaction by adding 10 µl per well of the Vivid® Substrate and $NADP^+$ mixture prepared in Step 5.1.7 and mix.

5.1.9 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 6.

2. **Endpoint Assay Mode:** Incubate the plate for the desired amount of time, then add 10 µl of recommended stop reagent (see Section 7.0) to each well to quench the reaction. Measure fluorescence in the fluorescent plate reader at excitation and emission wavelengths listed in Table 6.

   Note: Appropriate reaction times will vary by kit and experimental conditions. We recommend that you determine the linear activity range for the assay under the conditions you wish to use. Typically, such reaction times will fall within 5 to 60 minutes.

3. Proceed to Section 6.0 for data analysis.

Table 6. Recommended excitation and emission wavelengths and filter sets

		Vivid® Fluorescent Standard							
		Red		Blue		Green		Cyan	
Fluorescence Plate Readers	Excitation/ Emission	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width
with monochromators	excitation	530	--	409	--	485	--	400	--
	emission	585	--	460	--	530	--	502	--
using filters	excitation	530	25	405	20	485	20	405	40
	emission	605	55	460	40	530	25	490	40
with dichroic mirror	excitation	530	25	405	20	485	20	405	40
	emission	605	55	460	40	530	25	490	40
	dichroic	555	--	425	--	505	--	435	--

Red Standard is sodium salt of resorufin. Blue Standard is 3-cyano-7-hydroxycoumarin. Cyan Standard is 7-hydroxy-4-trifluoromethylcoumarin. We recommend exciting this dye off-peak at 400 nm (its excitation maximum is 385 nm) to minimize background from NADPH fluorescence. Green Standard is fluorescein.

For optimal signal to noise, filters must be blocked to OD of 6 outside their transparency range (UV and red blockage) and be free of pinholes. Filters may be purchased from:

Chroma Technology Corp.
72 Cotton Mill Hill, Unit A-9
Brattleboro, VT 05301
Phone: (800) 824-7662 or (802) 257-1800
Fax: (802) 257-9400.
www.chroma.com

6.0 SUGGESTED PROTOCOL FOR THE ANALYSIS OF RESULTS

6.1 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:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{rate in presence of test compound or positive inhibition control}}{\text{rate in absence of test compound or positive inhibition control}} \right) \text{X } 100\%$$

6.2 Endpoint Assay Mode

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

$$\% \text{ Inhibition} = \left(1 - \frac{\text{RFU in presence of test compound or positive inhibition control}}{\text{RFU in absence of test compound or positive inhibition control}} \right) \text{X } 100\%$$

Optional: Both types of data analysis above can be performed using a standard curve as described in Section 5.1.3 in order to calculate reaction rates as nmol product formed per unit time.

7.0 SUGGESTED CYP450 INHIBITORS (STOP REAGENT)

Table 7. Suggested CYP450 Inhibitors

Enzyme	Inhibitor (Stop Reagent)	Sigma-Aldrich Cat. no.	Suggested Final Concentration**
CYP1A2	α-naphthoflavone	N5757	3 µM
CYP2B6	miconazole	M3512	30 µM
CYP2C9	sulfaphenazole	S0758	10 µM
CYP2C19	miconazole	M3512	30 µM
CYP2D6	quinidine	Q3625	1 µM
CYP2E1	diethyldithiocarbamate	228680	100 µM
CYP3A4	ketoconazole	K1003	10 µM
CYP3A5	ketoconazole	K1003	30 µM

** To stop the reaction, the suggested final inhibitor concentration in the assay to produce inhibition of 90% or better is indicated in the above table. For an endpoint assay the volume of the added Stop Reagent should not exceed 10% of the final reaction volume [*e.g.,* 10 µl will be added per 100 µl reaction volume. This 10% increase in the volume of an endpoint reaction does not have a significant effect on the reaction (or the calculations)].

8.0 SOLVENT TOLERANCES

P450 activity can be inhibited by solvents commonly used to dissolve test compounds. While we always recommend including a solvent control in your experimental design, the following sample data is intended as a guide for the selection and use of organic solvents. Table values are percent inhibition at the indicated solvent concentration. Values preceded by a "+" indicate an increase in activity. Dashed lines indicate inhibition not detected. Note that lower concentrations are listed for 2E1 Blue; this isozyme is particularly sensitive to the presence of organic solvents.

Table 8. Solvent Tolerances

Vivid® Kit	Solvent concentration (%)	DMSO (% Inhibition)	Acetonitrile (% Inhibition)	Methanol (% Inhibition)	Ethanol (% Inhibition)
1A2 Blue	1	7	--	--	--
	0.1	--	--	--	--
	0.01	--	--	--	--
2B6 Blue	1	16	7	20	32
	0.1	--	--	--	--
	0.01	--	--	--	--
2B6 Cyan	1	--	--	--	9
	0.1	--	--	--	--
	0.01	--	--	--	--
2C9 Blue	1	55	9	46	61
	0.1	25	--	7	11
	0.01	--	--	--	--
2C9 Green	1	--	--	30	38
	0.1	--	--	--	--
	0.01	--	--	--	--
2C9 Red	1	21	5	45	53
	0.1	8	--	9	9
	0.01	5	--	--	--
2C19 Blue	1	23	--	21	42
	0.1	--	--	--	5
	0.01	--	--	--	--
2D6 Blue	1	58	--	37	56
	0.1	16	--	--	10
	0.01	4	--	--	--
2D6 Cyan	1	21	6	38	49
	0.1	--	--	4	5
	0.01	--	--	--	--
2E1 Blue	0.1	85	36	26	98
	0.01	35	15	3	75
	0.001	20	7	8	25
3A4 Blue	1	68	--	12	10
	0.1	25	--	4	--
	0.01	6	--	--	--
3A4 Cyan	1	68	4	20	11
	0.1	29	--	6	--
	0.01	7	--	--	--
3A4 Green	1	47	--	--	--
	0.1	9	--	--	--
	0.01	--	--	--	--
3A4 Red	1	48	--	6	5
	0.1	13	--	--	--
	0.01	--	--	--	--
3A5 Blue	1	75	5	29	32
	0.1	34	--	14	8
	0.01	8	--	5	6
3A5 Cyan	1	71	6	21	23
	0.1	30	--	8	6
	0.01	--	--	5	6
3A5 Green	1	15	--	--	--
	0.1	--	--	--	--
	0.01	--	--	--	--

9.0 REFERENCES

Cohen, L.H, *et al.* (2003) *In vitro* drug interactions of cytochrome P450: an evaluation of fluorogenic to conventional substrates. *Drug Metab. Dispos.* **31:**1005-15.

Marks, B.D., *et al.* (2002) A high throughput screening assay to screen for CYP2E1 metabolism and inhibition using a fluorogenic Vivid® P450 substrate. *Assay Drug Dev. Technol.* **1:**73-81.

Marks, B.D., *et al.* (2003) High-throughput screening assays for CYP2B6 metabolism and inhibition using fluorogenic Vivid® substrates. *AAPS PharmSci.* **5:**E18.

Marks, B.D, *et al.* (2004) High-throughput screening assays for the assessment of CYP2C9*1, CYP2C9*2, and CYP2C9*3 metabolism using fluorogenic Vivid® substrates. *J. Biomol. Screen.* **9:**439-49.

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.

For structures of the Vivid Substrates and poster presentations containing additional details and applications of Vivid CYP450 Screening Kits, please visit us online at: www.invitrogen.com/drugdiscovery.

10.0 PURCHASER NOTIFICATION

Limited Use Label License No. 162: Cytochrome P450 enzymes, assays and substrates

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