TECHNICAL MANUAL

P450-Glo™ Assays

Instructions for use of Products
V8421, V8422, V8751, V8752, V8761, V8762, V8771, V8772, V8781, V8782, V8791, V8792, V8801, V8802, V8811, V8812, V8881, V8882, V8891, V8892, V8901, V8902, V8911, V8912, V9001 AND V9002.

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P450-Glo™ Assays

All technical literature is available on the Internet at: www.promega.com/protocols/ Please visit the web site to verify that you are using the most current version of this Technical Bulletin. Please contact Promega Technical Services if you have questions on use of this system. E-mail: techserv@promega.com

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Promega Corporation · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA
Toll Free in USA 800-356-9526 · Phone 608-274-4330 · Fax 608-277-2516 · **www.promega.com**
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Part I: Introduction

1. Description

P450-Glo™ Assays[a–d] provide a luminescent method to measure cytochrome P450 (CYP) activity (1). The assays measure the activities of CYP enzymes from recombinant and native sources and test the effects of drugs and new chemical entities on CYP activities. All of these assays can be used for cell-free CYP inhibition studies. Many of these assays also can be used for cell-based CYP induction assays. The P450-Glo™ Substrates are CYP enzyme substrates that are derivatives of beetle luciferin [(4S)-4,5-dihydro-2-(6´-hydroxy-2´-benzothiazolyl)-4-thiazolecarboxylic acid]. The derivatives are converted by CYP enzymes to luciferin products. D-luciferin is formed and detected in a second reaction with the Luciferin Detection Reagent (Figure 1 and Table 1). The amount of light produced in the second reaction is proportional to CYP activity.

The P450-Glo™ Assays provide a luminogenic CYP substrate, a lyophilized Luciferin Detection Reagent and a reconstitution buffer. The user supplies a CYP preparation with the requisite buffer and NADPH, which is supplied by the NADPH Regeneration System (Cat.# V9510). In cell-based assays, NADPH in the cell is sufficient to support CYP activity. Protocols are configured for multiwell plate formats but can be easily adapted for single-tube applications. An overview of the protocol is provided in Figure 2.

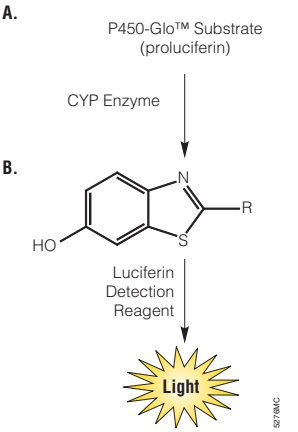

Figure 1. Conversion of P450-Glo™ substrate by cytochrome P450. CYP enzymes act on a luminogenic P450-Glo™ substrate (Reaction A) to produce a luciferin product that generates light with the Luciferin Detection Reagent (Reaction B), which is added after the CYP reaction has been completed. Cytochrome P450 substrate selectivity depends on the specific structure of the proluciferin substrate (Table 1). R varies as shown in Table 1.

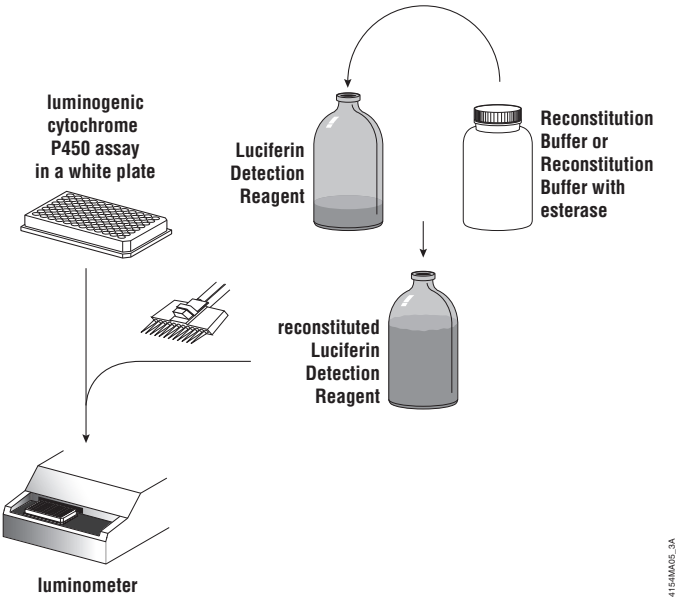

Figure 2. Flow diagram showing preparation and use of the reconstituted Luciferin Detection Reagent. Use the Reconstitution Buffer provided to reconstitute the lyophilized Luciferin Detection Reagent. Reconstitution Buffer for CYP2C19, CYP2D6 and CYP3A4/Luciferin-IPA assays contains esterase, and the Reconstitution Buffer for all other CYP assays does not contain esterase. **Label the blank space on the Luciferin Detection Reagent label with the appropriate CYP name to ensure the correct Luciferin Detection Reagent is used.**

The P450-Glo™ Assays are ideal for measuring:

- **CYP Inhibition:** Screen drugs and new chemical entities for inhibition of CYP activities in native or recombinant fractions.
- **Recombinant CYP Activity:** Measure recombinant CYP activities in membrane fractions from heterologous expression systems, such as insect cells and *E. coli.*
- **Native CYP Activity:** Measure native CYP activities in microsomal fractions from tissues (e.g., liver).
- **CYP Induction:** Identify and characterize inducers of CYP gene expression by measuring CYP activity within intact cells (e.g., hepatocytes).

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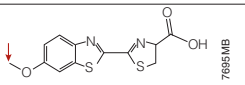
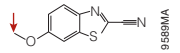
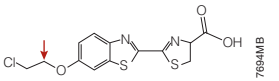
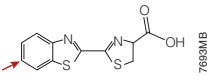
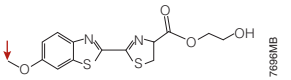
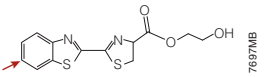
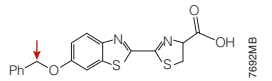
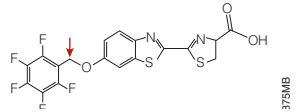
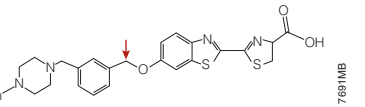
Table 1. Cytochrome P450 Enzymes, Recommended Substrates and Assay Formats.

Substrate[1]	P450 Enzyme Assays[2] (Biochemical Assays)	Cell-Based Assays
 Luciferin-ME	**CYP1A2, CYP2C8,** CYP2C9, CYP2J2, CYP4A11, CYP4F3B, CYP19	Not recommended
 Luciferin-1A2[3]	**CYP1A2**	CYP1A2 induction
 Luciferin-CEE	**CYP1A1**, **CYP1B1**, CYP3A7	CYP1A induction
 Luciferin-H	**CYP2C9**	CYP2C9 induction
 Luciferin-ME EGE	**CYP2D6**, CYP1A1, CYP1A2, CYP2B6[4]	Not recommended
 Luciferin-H EGE	**CYP2C19**, CYP1A1, CYP1A2	Not recommended
 Luciferin-BE	**CYP3A4**, CYP3A5, **CYP3A7**, CYP4F12	Not recommended
 Luciferin-PFBE	**CYP3A4**, CYP3A5, CYP3A7	CYP3A induction
 Luciferin-PPXE	**CYP3A4**, CYP3A5, CYP3A7	Not recommended

[1]The arrow indicates the site of modification by CYP.

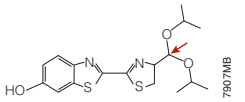
[2]The indicated substrate is provided with kits for the CYP shown in bold. These kits also can be used to assay the other CYP enzymes listed for a given substrate.

[3]The product of CYP1A2 and Luciferin-1A2 reaction is a luciferin precursor. D-luciferin is formed and detected by the Luciferin Detection Reagent supplemented with D-Cysteine.

[4]CYP2B6 activity was diminished when reactions were supplemented with cytochrome b5.

(continued, next page)

Table 1. Cytochrome P450 Enzymes, Recommended Substrates and Assay Formats. (continued)

Substrate[1]	P450 Enzyme Assays[2] (Biochemical Assays)	Cell-Based Assays
 Luciferin-IPA	**CYP3A4**	CYP3A induction

[1]The arrow indicates the site of modification by CYP.

[2]The indicated substrate is provided with kits for the CYP shown in bold. These kits also can be used to assay the other CYP enzymes listed for a given substrate.

Notes for Table 1

Four distinct substrates are available for the CYP3A enzymes.

Luciferin-IPA is the most sensitive and selective substrate for all CYP3A4 applications, including cell-free or cell-based inhibition assays and cell-based induction assays. Luciferin-IPA shows minimal cross-reactivity with CYP3A5 and 3A7 (Figure 6). The CYP3A4 reaction with Luciferin-IPA is only modestly inhibited by DMSO.

Luciferin-PPXE cross-reacts with CYP3A4, 3A5 and 3A7 (Figure 6). The CYP3A4 reaction with Luciferin-PPXE is highly insensitive to DMSO, with little or no inhibition at or below 0.25% DMSO.

Luciferin-PFBE is useful for cell-based CYP3A assays. For cell-free enzyme assays Luciferin-PFBE differs from Luciferin-BE in that Luciferin-PFBE is nonreactive with CYP4F12, background luminescence is typically lower than that with Luciferin-BE and its reaction with CYP3A4 is less sensitive to inhibition by DMSO than Luciferin-BE.

Luciferin-BE is the original luminogenic CYP3A substrate that cross-reacts with CYP3A5, 3A7 and 4F12. CYP3A4 reactions with Luciferin-BE are inhibited substantially by DMSO, so DMSO should be eliminated from reactions or kept at or below 0.1%.

1. Description (continued)

Advantages of the P450-Glo™ Assays include:

Speed: The luminescence format eliminates the need for time-consuming analyses such as liquid chromatograph/mass spec or thin-layer chromatography.

Simplified Method: Simple protocols make the assays amenable to high-throughput screening in multiwell plates.

Greater Sensitivity: Less CYP is required than in conventional methods because of enhanced sensitivity. This provides a cost-saving benefit and allows more accurate kinetic analysis.

No Fluorescence Interference: By using luminescence to monitor enzyme activity, the P450-Glo™ Assays obviate problems associated with fluorescent assays. In luminescent assays, there is no concern about the possible overlap between the fluorescent excitation and emission wavelengths of analytes, NADPH and CYP substrates. Such overlaps in fluorescent assays confound analysis and present misleading or irrelevant data.

Low False-Positive Rate: Use of a proprietary stabilized firefly luciferase (Ultra-Glo™ Luciferase) and a proprietary luciferase assay formulation minimizes the incidence of false positives due to luciferase inhibition.

Signal Stability: Glow-type luminescence provides a stable signal with a half-life of greater than 2 hours.

Cell Permeability: The substrates and reaction products of cell-based P450-Glo™ Assays are cell-permeant and amenable to a nonlytic format. This allows multiplexing with a cell viability assay so that cytochrome P450 activity can be normalized to the number of viable cells.

DMSO Tolerance: The P450-Glo™ reactions, except Luciferin-BE and Luciferin-PFBE with CYP3A4, are not inhibited substantially by DMSO at concentrations typically encountered (e.g., ≤0.25%).

2. Product Components and Storage Conditions

Product	Size	Cat.#
P450-Glo™ CYP1A1 Assay	10ml	V8751

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 70µl Luciferin-CEE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP1A1 Assay	50ml	V8752

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 350µl Luciferin-CEE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP1A2 Assay	10ml	V8771

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 200µl Luciferin-ME, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP1A2 Assay	50ml	V8772

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 1ml Luciferin-ME, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP1A2 Induction/Inhibition Assay	10ml	V8421

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 30µl Luciferin-1A2, 6mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer
- 1 × 100µl D-Cysteine, 500X

Product	Size	Cat.#
P450-Glo™ CYP1A2 Induction/Inhibition Assay	50ml	V8422

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 2 × 30µl Luciferin-1A2, 6mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer
- 1 × 100µl D-Cysteine, 500X

2. Product Components and Storage Conditions (continued)

Product	Size	Cat.#
P450-Glo™ CYP1B1 Assay	10ml	V8761

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 70µl Luciferin-CEE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP1B1 Assay	50ml	V8762

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 350µl Luciferin-CEE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP2C8 Assay	10ml	V8781

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 300µl Luciferin-ME, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP2C8 Assay	50ml	V8782

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 2 × 750µl Luciferin-ME, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP2C9 Assay	10ml	V8791

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 200µl Luciferin-H, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP2C9 Assay	50ml	V8792

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 1ml Luciferin-H, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP2C19 Assay	10ml	V8881

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 123µg Luciferin-H EGE
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer with esterase

Product	Size	Cat.#
P450-Glo™ CYP2C19 Assay	50ml	V8882

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 2 × 123µg Luciferin-H EGE
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer with esterase

Product	Size	Cat.#
P450-Glo™ CYP2D6 Assay	10ml	V8891

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 240µg Luciferin-ME EGE
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer with esterase

Product	Size	Cat.#
P450-Glo™ CYP2D6 Assay	50ml	V8892

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 900µg Luciferin-ME EGE
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer with esterase

2. Product Components and Storage Conditions (continued)

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay (Luciferin-IPA)	10ml	V9001

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 15µl Luciferin-IPA, 3mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer with esterase

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay (Luciferin-IPA)	50ml	V9002

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 60µl Luciferin-IPA, 3mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer with esterase

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay	10ml	V8801

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 100µl Luciferin-BE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay	50ml	V8802

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 500µl Luciferin-BE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay (Luciferin-PFBE) Cell-Based/Biochemical Assay	10ml	V8901

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 500µl Luciferin-PFBE, 2mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay (Luciferin-PFBE) Cell-Based/Biochemical Assay	50ml	V8902

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 3 × 500µl Luciferin-PFBE, 2mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO Tolerant Assay	10ml	V8911

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 15µl Luciferin-PPXE, 50mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO Tolerant Assay	50ml	V8912

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 2 × 15µl Luciferin-PPXE, 50mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP3A7 Assay	10ml	V8811

Each system contains sufficient reagents for 200 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 1 × 300µl Luciferin-BE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 10ml Reconstitution Buffer

Product	Size	Cat.#
P450-Glo™ CYP3A7 Assay	50ml	V8812

Each system contains sufficient reagents for 1,000 biochemical assays at 50µl per assay in 96-well plates. Includes:

- 2 × 750µl Luciferin-BE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml Reconstitution Buffer

2. Product Components and Storage Conditions (continued)

Storage Conditions: Store components at –20°C, except Luciferin-PPXE, which must be stored at –70°C. Store components protected from light. Store CYP enzyme preparations purchased separately at –70°C.

Luciferin-H EGE and Luciferin-ME EGE that are reconstituted in acetonitrile and the reconstituted Luciferase Detection Reagent can be stored at –20°C for up to 3 months. For convenience, the reconstituted Luciferin Detection Reagent can be stored at room temperature (approximately 23°C) without loss of activity for 24 hours or at 4°C for 1 week. Avoid multiple freeze-thaw cycles of components.

3. Overview of Cell-Free CYP Inhibition Assay Protocol

P450-Glo™Assays are performed in two steps (Figure 1).

Step 1. The CYP Reaction: A CYP enzyme and an appropriate substrate are combined in potassium phosphate (KPO_4) buffer with or without a test compound of interest, and the reaction is initiated by adding an NADPH regenerating system. Table 2 indicates the reaction components, final reagent concentrations and incubation times for Step 1. A convenient approach to assemble the CYP reactions is to prepare a 4X concentrated mixture of CYP enzyme, substrate and KPO_4 reaction buffer. A volume of this mixture representing one-fourth of the final reaction volume (e.g., 12.5µl in a 96-well plate) is combined with an equal volume of test compound solution to give one-half of the final reaction volume (e.g., 12.5µl added to bring the volume to 25µl in a 96-well plate). The reaction is initiated by adding 2X concentrated NADPH Regeneration System (Cat.# V9510) (e.g., 25µl added for a final volume of 50µl in a 96-well plate).

Note: "2X" and "4X" refers to a reagent that is prepared at two or four times the final reagent concentration, respectively.

Step 2. The Luciferin Detection Reaction: In this step, the luciferin product produced in Step 1 of the P450-Glo™ Assays is detected as a luminescent signal from a luciferase reaction. Step 2 is initiated by adding an equal volume of Luciferin Detection Reagent (e.g., 50µl added to a 50µl CYP reaction in a 96-well plate). This reagent simultaneously stops the CYP reaction and initiates a luminescent signal that is proportional to the amount of product formed in Step 1. Signals then are allowed to stabilize for 20 minutes at room temperature before reading luminescence on a luminometer.

Note: Do not use a fluorometer, which uses excitation light that will interfere with the luminescent readout.

Table 2. Reaction Components in the P450-Glo™ Assay.

Human CYP Preparation	CYP per Reaction (96-Well Plate)	KPO_4 Concentration	Substrate Concentration (K_m)	Incubation Time (37°C/RT)[1]
CYP1A1	0.5pmol	100mM	30µM Luciferin-CEE	10/30 minutes
CYP1A2	0.5pmol	100mM	100µM Luciferin-ME	10/30 minutes
CYP1A2	0.5pmol	100mM	6µM Luciferin-1A2	10/10 minutes
CYP1B1	1.0pmol	100mM	20µM Luciferin-CEE	20/30 minutes
CYP2C8	1.0pmol	50mM	150µM Luciferin-ME	30/45 minutes
CYP2C9	0.5pmol	25mM	100µM Luciferin-H	30/30 minutes
CYP2C19	0.25pmol	50mM	10µM Luciferin-H EGE	20/30 minutes
CYP2D6	0.25pmol	100mM	30µM Luciferin-ME EGE	30/45 minutes
CYP3A4	0.1pmol	100mM	3µM Luciferin-IPA[3]	10/10 minutes
CYP3A4	1.0pmol	200mM	50µM Luciferin-BE	30/30 minutes
CYP3A4	1.0pmol	200mM	50µM Luciferin-PFBE	10/30 minutes
CYP3A4	0.5pmol	200mM	25µM Luciferin-PPXE	15/30 minutes
CYP3A7	1.0pmol	100mM	150µM Luciferin-BE	30/30 minutes
Liver microsomes[2]	20µg	100mM	20–150µM substrate of choice	30/30 minutes
Liver microsomes with Luciferin-IPA	1µg	100mM	8µM	10/10 minutes
Liver microsomes with Luciferin-1A2	1µg	100mM	3µM	10/10 minutes

[1]RT = room temperature, which is defined as 20–25°C.

[2]Liver microsomes are not recommended for CYP1A2/Luciferin-ME, 2C8, 2C19 or 2D6 assays because of substrate cross-reactivity with other CYPs (Figure 6).

[3]Luciferin-IPA is the preferred substrate for cell-based CYP3A4 assays.

Part II: Biochemical Assays

4. Preparation of Buffers and Solutions

Materials to Be Supplied by the User
(Solution compositions are provided in Section 13.A.)

- 1M KPO_4 buffer (pH 7.4)
- distilled or deionized water
- active CYP preparation that includes CYP450 reductase (see Section 13.B for supplier information)
- preparation that lacks CYP activity for the minus-P450 control reactions
- acetonitrile (for CYP2D6 or CYP2C19 assays)
- NADPH Regeneration System (Cat.# V9510)
- 100mM Tris-HCl (pH 7.5) to dilute Luciferin-PPXE for CYP3A4 assays
- white opaque polystyrene nontreated flat-bottom multiwell plates (e.g., 96-well Costar® plates, Corning Cat.# 3912, or white 96 MicroWell® plates, Nunc Cat.# 236108). **Do not** use treated plates, black plates or clear plates.
- luminometer or charge-coupled device (CCD) capable of reading multiwell plates (If using a multifunctional reader be sure it is operating in the luminescence, not fluorescence, mode.)
- **Optional:** multichannel pipette or automated pipetting station

! For best results when performing assays with CYP2C8, 2C9, 3A4, 3A5 and 3A7, use a CYP preparation that is supplemented with cytochrome b5 in addition to CYP450 reductase.

4.A. Preparing the Reconstituted Luciferin Detection Reagent

1. Equilibrate the lyophilized Luciferin Detection Reagent and Reconstitution Buffer (supplied with CYP1A1, 1A2/Luciferin-ME, 1A2/Luciferin-1A2, 1B1, 2C8, 2C9, 3A4/Luciferin-BE, 3A4/Luciferin-PFBE, 3A4/Luciferin-PPXE and 3A7 assays) or Reconstitution Buffer with esterase (supplied with the CYP2C19, 2D6 and 3A4/Luciferin-IPA assays) to room temperature. For CYP1A2/Luciferin-1A2 assay, you also need to equilibrate the D-Cysteine, 500X, to room temperature.

   Note: There are three formulations of reconstituted Luciferin Detection Reagent, and all of them use the lyophilized Luciferin Detection Reagent. For CYP1A1, 1A2/Luciferin-ME, 1A2/Luciferin-1A2, 1B1, 2C8, 2C9, 3A4/Luciferin-BE, 3A4/Luciferin-PFBE, 3A4/Luciferin-PPXE and 3A7 assays, the Reconstitution Buffer is used. For CYP1A2/Luciferin-1A2 assay, the Reconstitution Buffer is used and the reconstituted Luciferin Detection Reagent is supplemented with D-Cysteine. For CYP2C19, 2D6 and 3A4/Luciferin-IPA assays, the Reconstitution Buffer with esterase is used.

! **The Reconstitution Buffer and Reconstitution Buffer with esterase are not interchangeable. Use the appropriate buffer for each assay. Label the bottle of reconstituted Luciferin Detection Reagent with the appropriate CYP name.**

2. Transfer the contents of one bottle of reconstitution buffer to the amber bottle containing the lyophilized Luciferin Detection Reagent. For the CYP1A2/Luciferin-1A2 assay, dilute the supplied D-Cysteine, 500X, to a final concentration of 1X in reconstituted Luciferin Detection Reagent. For 10ml (Part# V859A and V865A) and 50ml (Part# V859B and V865B) reconstituted Luciferin Detection Reagent, supplement with 20µl and 100µl of the supplied D-Cysteine, 500X, respectively. Mix by swirling or inverting several times to obtain a homogeneous solution. Store at room temperature until ready to use.

   Note: The reconstituted Luciferin Detection Reagent can be stored at room temperature for 24 hours or at 4°C for 1 week without loss of activity. For long-term storage, store at –20°C for up to 3 months. Be sure to mix the thawed Luciferin Detection reagent well before use.

4.B. Preparing the P450-Glo™ Luminogenic Substrates

There are many substrates available with the P450-Glo™ Assays for biochemical and cell-based assays. The indicated substrate is provided with kits for the CYP shown in bold in Table 1.

1. Luciferin-ME, Luciferin-CEE, Luciferin-H, Luciferin-BE and Luciferin-PFBE are provided as aqueous solutions. Thaw the substrate solutions, and keep on ice, protected from light. Store unused substrate at or below –20°C, protected from light.

   A precipitate may form in the Luciferin-ME upon freezing and thawing. Dissolve the precipitate by warming the vial to 37°C and vortex mixing.

   The Luciferin-BE substrate may appear viscous after thawing. Warm the vial briefly at 37°C, and vortex to reduce the viscosity.

2. Luciferin-1A2 and Luciferin-IPA are provided as a solution in DMSO. Thaw the solution, and keep at room temperature, protected from light, while setting up assays. Store unused substrates at or below –20°C, protected from light.

3. Luciferin-PPXE is provided as a 50mM stock solution in acidified DMSO (DMSO with 0.05N HCl). Thaw the substrate at room temperature, protected from light. Store the substrate at –70°C, protected from light.

4. Luciferin-H EGE and Luciferin-ME EGE are provided as dried pellets. Dissolve the pellet in acetonitrile to a final concentration of 10mM with vigorous mixing as follows:
   - To 123µg of Luciferin-H EGE, add 40µl of acetonitrile.
   - To 240µg of Luciferin-ME EGE, add 70µl of acetonitrile.
   - To 900µg of Luciferin-ME EGE, add 265µl of acetonitrile.

   Store unused substrate at –20°C, protected from light.

4.C. Preparing the 2X NADPH Regeneration System

Note: The NADPH Regeneration System (Cat.# V9510) is not supplied with the P450-Glo™ Assays and must be purchased separately. See Section 13.B for more information. The NADPH Regeneration System is **not required** for cell-based assays. In cell-based assays, NADPH in the cell is sufficient to support CYP activity.

Prepare the 2X NADPH regeneration system as directed in Tables 3 and 4. For CYP3A4 reactions with Luciferin-BE, Luciferin-PPXE or Luciferin-PFBE, prepare the 2X NADPH regeneration system with KPO_4 buffer as directed in Table 4. Use Tables 3 and 4 to calculate the volume of each component.

For 96-well plates, prepare 25µl of the appropriate 2X NADPH regeneration system for each well. Prepare the 2X NADPH regeneration system on the day of use, and store at room temperature until ready to use.

Table 3. Preparation of the 2X NADPH Regeneration System[1].

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
Luciferin-Free Water	22.0µl				
Solution A[1]	2.5µl				
Solution B[1]	0.5µl				
Final volume	**25.0µl**				

[1]For use in assays with Luciferin-ME, Luciferin-1A2, Luciferin-CEE, Luciferin-H, Luciferin-ME EGE, Luciferin-H EGE and Luciferin-IPA.

Table 4. Preparation of the 2X NADPH Regeneration System with KPO_4 Buffer[1].

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
Luciferin-Free Water	12.0µl				
1M KPO_4 buffer	10.0µl				
Solution A[2]	2.5µl				
Solution B[2]	0.5µl				
Final volume	**25.0µl**				

[1]For use in assays with Luciferin-BE, Luciferin-PPXE and Luciferin-PFBE.

[2]If you prefer to use purified NADPH in place of the 2X NADPH regeneration system, the 2X concentration of NADPH is 200µM.

4.D. Preparing the 4X CYP Reaction Mixture

The volumes recommended here are for 50µl CYP reactions in 96-well plates. For smaller well formats, scale volumes as necessary. Thaw CYP preparations immediately before use to minimize enzyme instability.

Note: We suggest dispensing the membranes into smaller volumes and storing at –70°C to minimize freeze/thaws.

1. Thaw the CYP preparation rapidly at 37°C, then place on ice.
2. Prepare 12.5µl of 4X CYP reaction mixture for each CYP reaction in a 96-well plate. Reaction mixtures include the CYP enzyme, its luminogenic substrate and KPO_4 buffer. For CYP3A4 assays with Luciferin-BE, Luciferin-PPXE or Luciferin-PFBE, prepare 4X CYP reaction mixtures without KPO_4 buffer to avoid substrate precipitation. For these assays, the KPO_4 is provided as a component of the 2X NADPH regeneration system (Section 4.C).

   Use the concentration of each component listed in Table 5. When preparing 4X CYP reaction mixtures with substrates other than Luciferin-PPXE, use water to bring the reaction mixture to the final volume. When preparing 4X CYP reaction mixtures with Luciferin-PPXE, use 100mM Tris-HCl (pH 7.5) to enhance Luciferin-PPXE solubility. Mix well after each component is added, and add the CYP enzyme last. The membranes in the CYP preparation may settle to the bottom of the tube, so it may be necessary to mix before dispensing. Store the 4X CYP reaction mixture on ice until ready to use.
3. For minus-P450 control reactions to measure background, prepare a 4X control reaction mixture using an equivalent amount of protein from a membrane preparation that lacks CYP activity. Prepare 12.5µl of 4X control reaction mixture for each minus-P450 control reaction in a 96-well plate. The membranes in the control preparation may settle to the bottom of the tube, so it may be necessary to mix before dispensing. Store mixture on ice until ready to use.

4.D. Preparing the 4X CYP Reaction Mixture (continued)

Table 5. Components of the 4X CYP Reaction Mixtures.

CYP Isoform	Amount of CYP/12.5µl[1]	KPO_4 Concentration	Substrate Concentration
CYP1A1	0.5pmol	400mM	120µM Luciferin-CEE
CYP1A2	0.5pmol	400mM	400µM Luciferin-ME
CYP1A2	0.5pmol	400mM	24µM Luciferin-1A2
CYP1B1	1.0pmol	400mM	80µM Luciferin-CEE
CYP2C8	1.0pmol	200mM	600µM Luciferin-ME
CYP2C9	0.5pmol	100mM	400µM Luciferin-H
CYP2C19	0.25pmol	200mM	40µM Luciferin-H EGE
CYP2D6	0.25pmol	400mM	120µM Luciferin-ME EGE
CYP3A4	0.1pmol	400mM	12µM Luciferin-IPA
CYP3A4	1.0pmol	See note 2.	200µM Luciferin-BE
CYP3A4	1.0pmol	See note 2.	200µM Luciferin-PFBE
CYP3A4	0.5pmol	See note 2.	100µM Luciferin-PPXE
CYP3A7	1.0pmol	400mM	600µM Luciferin-BE[3]
Liver microsomes[4]	20µg	400mM	80–600µM substrate of choice
Liver microsomes with Luciferin-IPA	1µg	400mM	32µM Luciferin-IPA
Liver microsomes with Luciferin-1A2	1µg	400mM	12µM Luciferin-1A2

[1]Add the recommended amount of CYP to a solution containing 4X concentrated substrate and KPO_4 buffer (i.e., 4X CYP reaction mixture) for a final volume of 12.5µl. This 4X CYP reaction mixture becomes 1X when included in a 50µl final reaction volume. For smaller reaction volumes, scale the amount as necessary.

[2]Substrate precipitation is avoided when the KPO_4 buffer is withheld from the 4X CYP reaction mixture and is added at a 2X concentration (400mM) as a component of the 2X NADPH regeneration system (Section 4.C).

[3]To avoid substrate precipitation when preparing the 4X CYP3A7 reaction mixture, add the Luciferin-BE substrate last.

[4]For CYP1A2/Luciferin-ME, 2C8, 2C19 and 2D6 assays, the best results are obtained with a recombinant or purified CYP preparation; we do not recommend use of liver microsomes with the substrates for these CYPs because of cross-reactivity with other

4.E. Suggested Plate Layout with Controls

The suggested layout for a 96-well plate format is shown in Figure 3.

- **Minus-P450 Control:** This control will give a measure of the CYP-independent background signal present in the assay. It contains a luminogenic P450-Glo™ substrate, KPO_4 buffer and NADPH regeneration system but no CYP. For example, a recombinant CYP membrane fraction is replaced with an equivalent fraction that lacks CYP expression. The average of the minus-P450 control values is subtracted from the luminescence of the CYP reactions to give the net CYP-dependent luminescence.
- **Control Inhibitor:** This control determines the capacity of the system to detect inhibition by test compounds. It contains an active CYP preparation, luminogenic P450-Glo™ substrate, KPO_4 buffer and known inhibitor.
- **Untreated:** Values from these wells represent total CYP activity. They contain an active CYP preparation, KPO_4 buffer and luminogenic P450-Glo™ substrate without a known inhibitor or test compound.
- **TC 1–TC 29:** Luminescent values from these wells are compared to values from untreated control wells to ascertain the effect of the test compounds on CYP activity. They contain an active CYP preparation, luminogenic P450-Glo™ substrate, KPO_4 buffer and test compound (TC). A typical first-pass screening concentration of test compounds is 10µM. Alternatively, a range of concentrations of compounds can be tested to measure an IC_{50} value.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Minus P450 Control	Minus P450 Control	Minus P450 Control	TC 6	TC 6	TC 6	TC 14	TC 14	TC 14	TC 22	TC 22	TC 22
B	Control Inhibitor	Control Inhibitor	Control Inhibitor	TC 7	TC 7	TC 7	TC 15	TC 15	TC 15	TC 23	TC 23	TC 23
C	Untreated	Untreated	Untreated	TC 8	TC 8	TC 8	TC 16	TC 16	TC 16	TC 24	TC 24	TC 24
D	TC 1	TC 1	TC 1	TC 9	TC 9	TC 9	TC 17	TC 17	TC 17	TC 25	TC 25	TC 25
E	TC 2	TC 2	TC 2	TC 10	TC 10	TC 10	TC 18	TC 18	TC 18	TC 26	TC 26	TC 26
F	TC 3	TC 3	TC 3	TC 11	TC 11	TC 11	TC 19	TC 19	TC 19	TC 27	TC 27	TC 27
G	TC 4	TC 4	TC 4	TC 12	TC 12	TC 12	TC 20	TC 20	TC 20	TC 28	TC 28	TC 28
H	TC 5	TC 5	TC 5	TC 13	TC 13	TC 13	TC 21	TC 21	TC 21	TC 29	TC 29	TC 29

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Figure 3. Plate layout for the P450-Glo™ Assays.

5. Protocol for Performing Biochemical Assays

The following approach can be used to study the effects of test compounds on the activity of a CYP enzyme of interest. Use the suggested plate layout shown in Figure 3.

The final volume of each CYP reaction will be 50µl in a standard 96-well plate. Reagent volumes are given for individual wells of a 96-well plate.

The amounts of CYP recommended in Table 5 should provide strong signals. Use Figure 7 as a guide if you prefer to use more or less enzyme.

1. Add up to 12.5µl of test compound per well of a 96-well plate. If the volume of test compound is less than 12.5µl, add water to bring the volume of each well to 12.5µl. Add 12.5µl of water or test compound vehicle to the "untreated" and "minus-P450 control" wells.

   Note: Organic solvent should be kept to a minimum to avoid potential effects on CYP activities. Importantly, DMSO is a known inhibitor of many CYP3A4 reactions (2). For CYP3A4 reactions with Luciferin-BE and Luciferin-PFBE, the final concentration of DMSO should not exceed 0.1%. CYP3A4 reactions with Luciferin-PPXE and Luciferin-IPA show little or no sensitivity to DMSO at or below 0.25%. None of the P450-Glo™ reactions are substantially affected by acetonitrile, methanol or ethanol at or below 1.0%, and the nonCYP3A4 assays are not affected by DMSO at or below 1%.

2. **CYP reactions:** Add 12.5µl of the 4X CYP reaction mixture (prepared in Section 4.D) to each well. Mix gently.

   Minus-P450 control reactions: Add 12.5µl of the 4X control reaction mixture prepared with a fraction that lacks CYP activity (prepared in Section 4.D) to each well. Mix gently.

3. Pre-incubate the plate at 37°C or room temperature for 10 minutes.

   Note: See Section 9.C for a discussion of CYP reaction time and temperature.

4. Start CYP reactions by adding 25µl of the appropriate 2X NADPH regeneration system to the CYP assays and minus-P450 control reactions. Mix briefly.

5. Incubate the plate at the same temperature used during the pre-incubation step (Step 3) for 10–45 minutes. See Table 2 for recommended incubation times.

6. Add 50µl of reconstituted Luciferin Detection Reagent to the CYP assays and control reactions.

(!) The Luciferin Detection Reagent is prepared with Reconstitution Buffer or Reconstitution Buffer with esterase. For CYP1A2/Luciferin-1A2 assay, the Luciferin Detection Reagent is prepared with Reconstitution Buffer and supplemented with D-Cysteine. See Section 4.A. These formulations are not interchangeable. Use the appropriate reagent for each assay.

7. Mix the plate for 10 seconds on an orbital shaker or by gently tapping the plate.
8. Incubate the plate at room temperature for 20 minutes to stabilize the luminescent signal.
9. Record luminescence using a luminometer or CCD camera.

   Note: Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Do not use a fluorometer. Do not use filters with the luminometer.

6. Results

Calculate the net luminescence of each CYP assay by subtracting the luminescence of the average of the minus-P450 control wells. Net signals from CYP reactions that do not include a test compound represent total CYP activity. Changes in net signal from test compound-treated samples as compared to untreated control samples represent modulation of CYP activity by the test compound. Changes typically will be seen as decreases due to CYP inhibition. However, some test compounds may increase signal because they exhibit positive cooperativity with the P450-Glo™ substrate. This phenomenon has been frequently reported for CYP2C9 and 3A4 (3,4).

7. Quantifying P450-Glo™ Signals with D-Luciferin Standard Curves

The concentration of D-luciferin generated by CYP in P450-Glo™ Assays can be determined by comparing luminescence from CYP reactions to that from a D-luciferin standard curve. The range of D-luciferin concentrations generated in P450-Glo™ Assays is in the linear portion of the standard curve for D-luciferin as illustrated in Figure 4. Standard curve measurements should be performed at the same time and in the same plate as samples. Use the plate layout shown in Figure 5. By comparing signals from CYP reactions to those from D-luciferin standards, the quantity of D-luciferin generated by CYP can be determined.

See Section 7.B for an explanation of why interpolated values for Luciferin-PPXE reactions are multiplied by two.

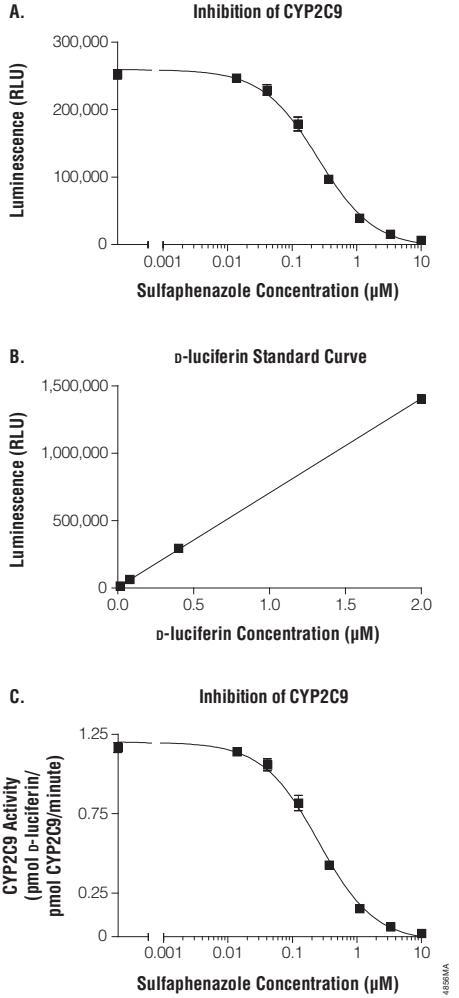

Figure 4. Representative P450-Glo™ Assay data. CYP2C9 reactions were performed in the presence or absence of the CYP2C9 inhibitor, sulfaphenazole, as described in Section 5. **Panel A.** Inhibition of CYP2C9 by sulfaphenazole is expressed in terms of relative light units (RLU). **Panel B.** A D-luciferin standard curve was performed in parallel with CYP2C9 reactions as described in Section 7.A and analyzed by linear regression ($r^2 = 0.99$). **Panel C.** Luminescent signals from CYP2C9 reactions were compared to those from the D-luciferin standard curve to interpolate the D-luciferin concentrations. D-luciferin concentrations then were used to calculate CYP2C9 reaction rates (pmol D-luciferin/pmol CYP2C9/minute). The IC_{50} value derived from Panel A or C is 0.2µM. Luminescence was measured using a POLARstar luminometer (BMG Labtech).

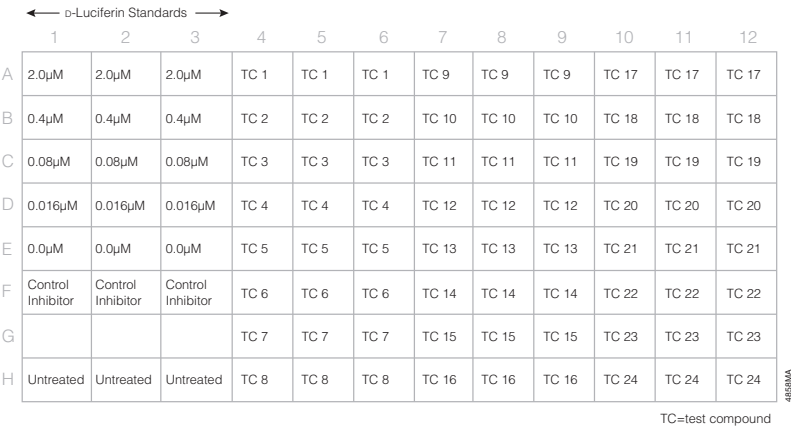

Figure 5. Plate layout for assays with a D-luciferin standard curve.

7.A. Generating a D-Luciferin Standard Curve

Prepare D-luciferin stock solutions and D-luciferin standards at a location separate from where the P450-Glo™ Assays are performed. **Because of the sensitivity of the luciferase reaction, even small amounts of luciferin contamination can affect assay results.** This protocol is written for a 96-well plate format. For smaller well formats, scale reagent volumes as necessary.

1. To prepare D-luciferin standards, dissolve 5mg of Beetle Luciferin, Potassium Salt (Cat.# E1601), in 7.85ml of water to make a 2mM stock solution of D-luciferin.
2. Add 40µl of 2mM D-luciferin to 960µl of water to make an 80µM working stock solution.
3. Prepare the 4X D-luciferin standards:
   i. Label four tubes: 8µM, 1.6µM, 0.32µM and 0.064µM, respectively.
   ii. Pipette 900µl of water into the 8µM tube and 800µl water into the other three tubes.
   iii. Add 100µl of the 80µM D-luciferin working stock prepared in Step 2 to the 8µM tube. Mix thoroughly by pipetting.
   iv. Transfer 200µl from the 8µM tube to the 1.6µM tube. Mix thoroughly by pipetting.
   v. Transfer 200µl from the 1.6µM tube to the 0.32µM tube. Mix thoroughly by pipetting.
   vi. Transfer 200µl from the 0.32µM tube to the 0.064µM tube. Mix thoroughly by pipetting.

   Note: Store the D-luciferin stock solutions at –20°C.

7.A. Generating a D-Luciferin Standard Curve (continued)

4. Prepare the 4X CYP reaction mixture, 4X control reaction mixture and 2X NADPH regeneration system as described in Section 4. Prepare enough 4X control reaction mixture for all standards. **Also, be sure to add the appropriate P450-Glo™ substrate to the 4X control reaction mixture.**

5. Add 12.5µl of 4X D-luciferin standards to the appropriate wells (8µM standards to wells labeled 2µM, 1.6µM standards to wells labeled 0.4µM, 0.32µM standards to wells labeled 0.08µM, and 0.064µM to wells labeled 0.016µM). Add 12.5µl of water to 0µM D-luciferin wells. **Take care to avoid cross-contaminating the wells with D-luciferin.**

   Note: The luminescence from the sample labeled as 0µM is equivalent to the minus-P450 control in Figure 3.

6. Add 12.5µl of the 4X control reaction mixture to the 2µM, 0.4µM, 0.08µM, 0.016µM and 0µM standard wells.

7. Set up wells with control and test compounds, and proceed with the assay as described in Section 5.

7.B. Data Analysis

- Subtract the average luminescence of the 0µM D-luciferin standard wells from all luminescence values (including 0µM D-luciferin).

- Perform linear regression analysis of luminescence from standards to generate a standard curve, where X represents the D-luciferin concentration and Y represents the luminescence (in relative light units, RLU).

- Interpolate CYP-generated D-luciferin concentrations in test samples by comparing their RLU values to those of the standard curve.

- To convert D-luciferin concentrations to a CYP reaction rate, consider the interpolated D-luciferin concentration, reaction volume, incubation time and amount of CYP assayed. For example, a 30-minute reaction with 1pmol of CYP generates 1µM D-luciferin. In a 50µl reaction volume, 1µM D-luciferin is 50pmol. The activity is 50pmol D-luciferin/pmol CYP/30 minutes or 1.67pmol D-luciferin/pmol CYP/minute.

- The luminescence from all samples should not be higher than that of the 2µM standard. If any values exceed that of the highest standard, the range of the standard curve can be extended by including standards at higher concentrations (e.g., 10µM and 50µM D-luciferin).

(!) Luciferin concentrations interpolated for Luciferin-PPXE reactions are half of their true values because Luciferin-PPXE is provided as a 50:50 mixture of D- and L-forms and the Luciferin Detection Reagent only detects D-luciferin. The rate of metabolism by CYP enzymes of D-luciferin-PPXE and L-luciferin-PPXE are equal, so the reaction product is a 50:50 mixture of D-luciferin and L-luciferin. **To calculate the true values, multiply interpolated values by two.**

8. K_m Measurements

The K_m value for a given CYP may vary somewhat between enzyme preparations (5). The concentrations of P450-Glo™ substrates recommended here are representative K_m concentrations for recombinant CYP enzyme preparations. When measuring K_m values, Luciferin-H, Luciferin-ME, Luciferin-CEE, Luciferin-H EGE and Luciferin-ME EGE cause a partial inhibition of luciferase at the upper end of the concentration ranges tested and thus diminish the brightness of the detection step. Such luciferase inhibition is not observed with Luciferin-BE, Luciferin-PFBE, Luciferin-PPXE, Luciferin-IPA or or Luciferin-1A2. Without compensating for luciferase inhibition by the former substrates, the system is less sensitive to detect luciferin at the high end of the substrate concentration range than at the low end, resulting in an underestimate of the K_m value. For the K_m values of reactions with Luciferin-ME, Luciferin-CEE, Luciferin-H, Luciferin-H EGE and Luciferin-ME EGE reported in Table 2, compensation for luciferase inhibition was made by performing CYP reactions at a range of substrate concentrations, stopping the reaction by adding the reconstituted Luciferin Detection Reagent, then adjusting the substrate concentration in all reactions to the highest concentration in the range. In this way the sensitivity of luciferase to detect CYP-generated luciferin was equalized across the range of substrate concentrations. No compensation was made for the substrate consumed during the CYP reaction because less than 1% of the total substrate was consumed. K_m values measured using this method were in good agreement with values determined by integration of a luciferin peak using HPLC (data not shown).

9. General Considerations

9.A. Substrate Specificity

Some P450-Glo™ substrates have enhanced CYP enzyme selectivity over many conventional substrates. However, different CYP enzymes can react with more than one P450-Glo™ substrate (Figure 6). For best results, we recommend the enzyme and substrate combinations shown in Tables 1 and 2.

9.B. Cytochrome P450 Concentration

Although it is necessary to use enough CYP enzyme to generate a detectable amount of luciferin, large amounts of protein or phospholipid from microsome preparations can bind nonspecifically to a drug or inhibitor, leading to a reduction in the effective concentration and overestimation of K_m and K_i values (6). General recommendations for the amount of CYP are made in Table 2. CYP concentrations can be increased for brighter signals or reduced further as a cost-saving measure or to reduce nonspecific binding. The enzyme titration curves shown in Figure 7 can be used as a guide if you prefer to use more or less enzyme.

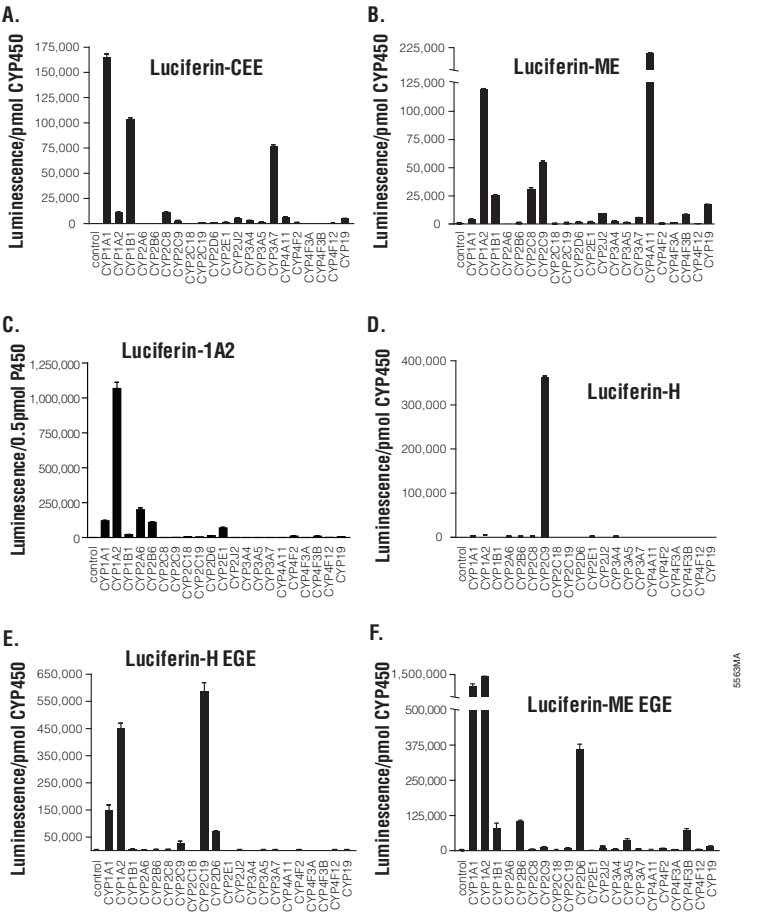

Figure 6. Selectivity of the P450-Glo™ substrates for human CYP enzymes. Recombinant CYP enzymes in insect cell microsomes were assayed for 30 minutes. All CYPs were co-expressed with CYP450 reductase, and some were supplemented with cytochrome b5 (CYP2A6, 2C8, 2C9, 2C19, 2E1, 2J2, 3A4, 3A5, 3A7 and all 4F enzymes). Control reactions used insect cell microsomes devoid of CYP activity. Luciferin-1A2 reactions were performed for 10 minutes with 6µM substrate. Luciferin-IPA reactions were performed for 10 minutes with 3µM substrate. All other reactions were performed for 30 minutes with 50µM substrate. **Panel A.** Luciferin-CEE major activities: CYP1A1, 1B1 and 3A7. **Panel B.** Luciferin-ME major activities: CYP1A2, 1B1, 2C8, 2C9 and 4A11. **Panel C**. Luciferin-1A2 major activity: CYP1A2. **Panel D.** Luciferin-H major activity: CYP2C9. **Panel E.** Luciferin-H EGE major activities: CYP2C19, 1A1 and 1A2. **Panel F.** Luciferin-ME EGE major activities: CYP2B6, 2D6, 1A1 and 1A2.

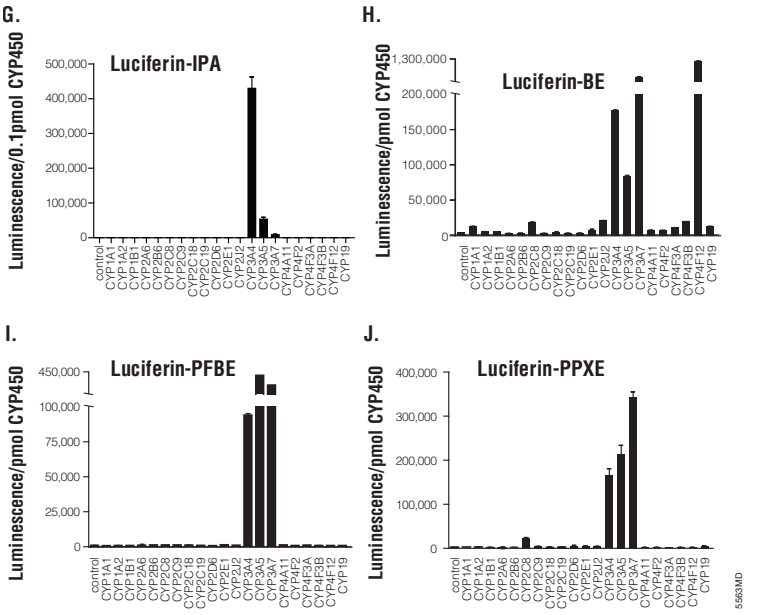

Figure 6. Selectivity of the P450-Glo™ substrates for human CYP enzymes (continued). Panel G. Luciferin-IPA major activity: CYP3A4. **Panel H.** Luciferin-BE major activities: CYP3A4, 3A5, 3A7 and 4F12. **Panel I.** Luciferin-PFBE major activities: CYP3A4, 3A5 and 3A7. **Panel J.** Luciferin-PPXE major activities: CYP3A4, 3A5 and 3A7.

9.C. Assay Time and Temperature

CYP reactions are generally performed at 37°C, but they also may be performed at room temperature (20–25°C) as shown in Figure 8. The suggested incubation times (Table 2) give strong signals within the linear range of the assays (Figure 8). If you prefer a different incubation time, refer to Figure 8 to determine if a shorter time gives adequate signal or if a longer time is within the linear range.

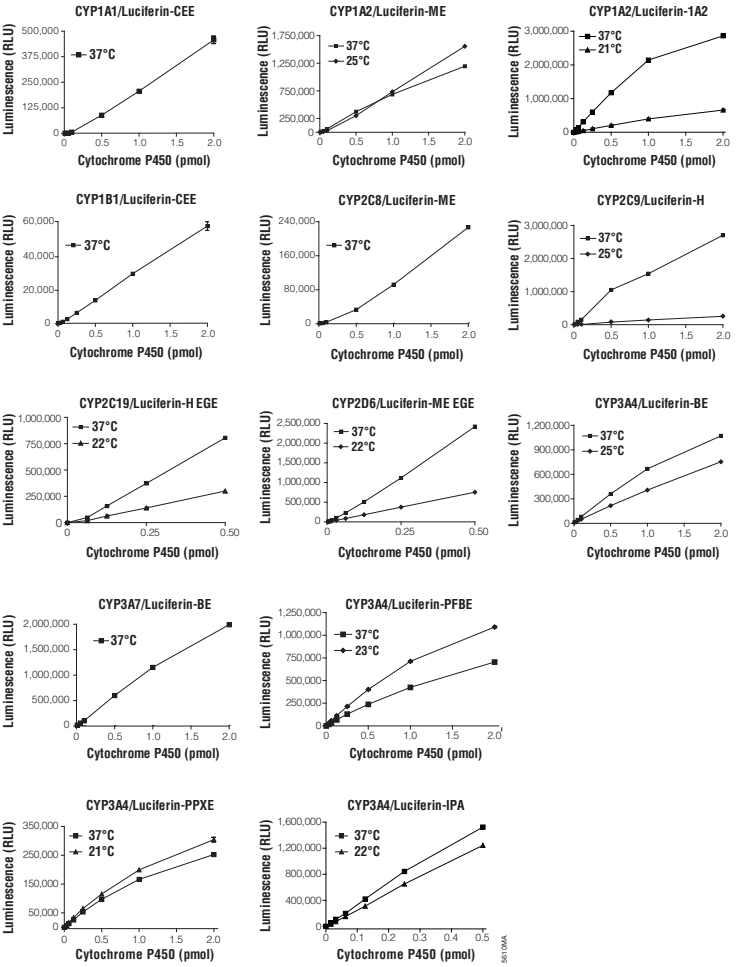

Figure 7. Titration of CYP. P450-Glo™ Assays were performed with a range of CYP concentrations. Substrate concentrations and incubation times were as recommended in Table 2.

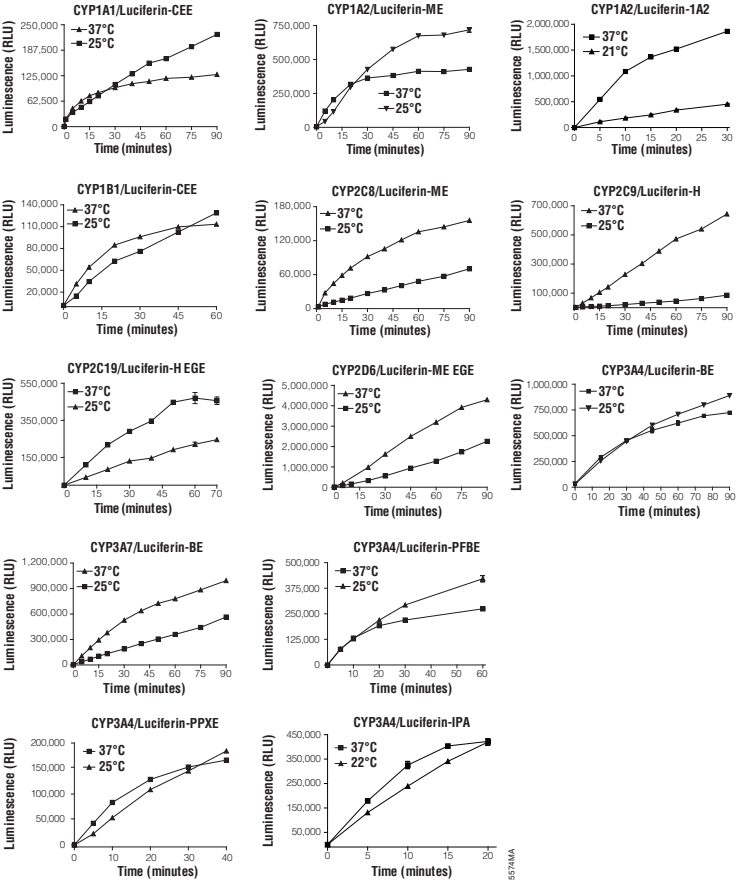

Figure 8. Incubation time and temperature. P450-Glo™ reactions (50µl) were performed with the enzyme and substrate concentrations indicated in Table 2. Reactions were incubated at room temperature (20–25°C) or 37°C for up to 90 minutes before adding reconstituted Luciferin Detection Reagent.

Part III: Cell-Based Assays

10. Protocol for Performing Cell-Based Assays

The P450-Glo™ substrates and reaction products are cell-permeant. This allows development of cell-based assays (1). In these assays, a luminogenic substrate is incubated with cultured cells for an appropriate period of time. Intracellular CYP enzymes convert the substrate to luciferin product, which passes out of cells and then can be detected with the Luciferin Detection Reagent. The luciferin produced is measured using either a nonlytic assay (perform the P450-Glo™ Assay with an aliquot of intact-cell supernatant) or a lytic assay (perform the P450-Glo™ Assay in a well containing cells). In either type of assay, the light output of the luciferase reaction is proportional to CYP activity. In a nonlytic assay, after an aliquot of the reaction is removed, the remaining cells can be used for additional cell-based testing; for example, a cell viability assay may be run to normalize CYP activities to viable cell number (e.g., CellTiter-Glo® Luminescent Cell Viability Assay; Section 13.D).

Cell-based applications of P450-Glo™ Assays include measurements of basal CYP activities, induction of these activities by test compounds and inhibition of both basal and induced CYP activities by test compounds. CYP inductions also can be observed as marker events to detect nuclear receptor and aryl hydrocarbon receptor ligands as exemplified by the following:

- CYP3A/Luciferin-IPA and CYP3A/Luciferin-PFBE activities are induced by ligands for the pregnane X receptor (PXR), constitutive androstane receptor (CAR) and glucocorticoid receptor (GR).
- CYP1A2/Luciferin-1A2 activity is induced by ligands for the aryl hydrocarbon receptor (AHR).
- CYP1A/Luciferin-CEE activity is induced by ligands for the aryl hydrocarbon receptor (AHR).

Chemically mediated CYP gene inductions have been observed in numerous cultured cell types. Transcriptional inducers of CYP genes cause increased CYP enzyme levels, which can be measured using the P450-Glo™ Assay. The following protocols relate specifically to assays of monolayer hepatocyte cultures; however the general methods are applicable to other cell types that express CYPs (e.g., HepaRG, HepG2, MCF7, FA2N-4, DPX-2). Cell-based CYP induction assays are typically performed with cells grown in monolayers. With hepatocytes, it is essential to follow a well defined protocol to maintain CYP expression and an inducible phenotype. The P450-Glo™ Assay is used to measure gene induction in hepatocytes cultured according to the methods described in Sections 11.B and 11.C, and we expect that other established culture/induction schemes can be followed (7,8). The highly sensitive Luciferin-IPA substrate also can be used to detect robust basal CYP3A activities in hepatocyte suspension cultures (Section 10.D).

10.A. Measuring CYP Activity in Cultured Cells

The P450-Glo™ Assays recommended for cell-based applications are:

- P450-Glo™ CYP3A4 Assay with Luciferin-IPA (Cat.# V9001, V9002) and P450-Glo™ CYP3A4 Assay with Luciferin-PFBE (Cat.# V8901, V8902)

  These assays can be used to selectively measure CYP3A activity in hepatocytes. Significant activities of other human CYPs have not been detected with Luciferin-IPA or Luciferin-PFBE.

- P450-Glo™ CYP1A2 Assay with Luciferin-1A2 (Cat.# V8421, V8422)

  This assay can be used to measure CYP1A2 activity in hepatocytes. Luciferin-1A2 is highly selective for CYP1A2.

- P450-Glo™ CYP2C9 Assay with Luciferin-H (Cat.# V8791, V8792)

  This assay can be used to measure CYP2C9 activity in human hepatocytes and other human cell types. Luciferin-H is highly selective for CYP2C9.

- P450-Glo™ CYP1A1 Assay (Cat.# V8751, V8752) and P450-Glo™ CYP1B1 Assay (Cat.# V8761, V8762) with Luciferin-CEE.

  The P450-Glo™ CYP1A1 Assay can be used to selectively measure CYP1A1 activity in hepatocytes where cross-reacting CYP1B1 and CYP3A7 are absent. However, CYP1A1 and CYP1B1 assays can detect a mixture of CYP1A1 and CYP1B1 activities in cell types that express both enzymes.

Consider including an inhibition control for induced P450 activity with a selective P450 inhibitor, such as 5µM α-naphthoflavone for CYP1A2/Luciferin-1A2, 2µM sulfaphenazole for CYP2C9/Luciferin-H, and 1µM ketoconazole for CYP3A4/Luciferin-IPA. The inhibition control will confirm that the induced activity is from the desired CYP enzyme. Please refer to Figure 6 for the P450-Glo™ substrate selectivities.

Note: The NADPH Regeneration System (Cat.# V9510) is not supplied with the P450-Glo™ Assays and must be purchased separately. See Section 13.B for more information. The NADPH Regeneration System is **not required** for cell-based assays. In cell-based assays, NADPH in the cell is sufficient to support CYP activity.

10.B. Nonlytic P450-Glo™ Assays Using Cultured Cells in Monolayers

Reserve some empty wells for background measurements. Recommendations for cell culture conditions are given in Sections 11.B and 11.C.

Optional: If you intend to combine the P450-Glo™ Assay with a luminescent cell viability assay, consider culturing cells on white-walled, collagen-coated plates with clear bottoms (e.g., BD BioCoat™ plates, BD Biosciences Cat.# 354650).

1. Treat cells with test compounds. For CYP gene induction studies, cells are typically treated with inducers for 24–72 hours. Optimal treatment time should be determined empirically; however, 48 hours is a common starting point. See Section 11.A for examples of CYP gene inducers that can be used as positive controls. Change medium with test compounds, etc., once daily for the duration of the treatment time.

10.B. Nonlytic P450-Glo™ Assays Using Cultured Cells in Monolayers (continued)

2. For **CYP3A4/Luciferin-IPA, CYP3A4/Luciferin-PFBE**, **CYP2C9/Luciferin-H**, **CYP1A1/Luciferin-CEE**, and **CYP1B1/Luciferin-CEE** assays, replace culture medium with fresh medium containing a luminogenic CYP substrate after the experimental treatment as indicated in Table 6. See Note 1.

   Optional: Wash cells with medium or phosphate buffered saline before adding medium with substrate. Some compounds that induce CYP gene expression also inhibit the CYP enzyme activity that has been induced. To observe induction, you may need to remove the inducer by including a wash step prior to adding a luminogenic substrate.

   For **CYP1A2/Luciferin-1A2** assays, remove the culture medium completely after treatment and wash cells twice with two volumes of Krebs-Henseleit buffer or PBS. Add diluted Luciferin-1A2 in Krebs-Henseleit Buffer (e.g., Sigma Cat.# K3753) or phosphate buffered saline (PBS). This P450-Glo™ Assay cannot be performed in most cell culture media because they contain L-cysteine, which interferes with the CYP1A2/Luciferin-1A2 assay.

   Notes:

   1. See Section 4.B for substrate preparation instructions.
   2. If inhibitors of basal or induced CYP enzyme activity are being tested, add them at this point with the luminogenic substrate.
   3. Salicylamide is added to inhibit phase II conjugation of the CYP product. Prepare a fresh 3M salicylamide stock in DMSO from the powder every time. When diluting salicylamide in Krebs-Henseleit Buffer or PBS, a white "precipitate" of salicylamide may occur. With mixing, all of the salicylamide will dissolve in the Krebs-Henseleit Buffer or PBS.

3. To determine background luminescence, add luminogenic substrate in medium to a set of empty wells (no cells). For CYP1A2/Luciferin-1A2 assay, add Luciferin-1A2 in the Krebs-Henseleit Buffer or PBS containing 3mM salicylamide to a set of empty wells (no cells).

4. Incubate plates at 37°C for 30–60 minutes for Luciferin-IPA and Luciferin-1A2, or 3–4 hours for all other substrates as indicated in Table 6.

5. During the incubation prepare the Luciferin Detection Reagent by transferring the entire contents of the bottle of the appropriate reconstitution buffer to the amber bottle containing Luciferin Detection Reagent. For the CYP1A2/Luciferin-1A2 assay, add the supplied D-cysteine, 500X, to the reconstituted Luciferin Detection Reagent to a final concentration of 1X. For 10ml (Part# V859A and V865A) and 50ml (Part# V859B and V865B) reconstituted Luciferin Detection Reagent, add 20µl and 100µl of the supplied D-cysteine, 500X, resepectively. Mix by swirling or inverting several times, and equilibrate to room temperature before use. Store the unused portion at –20°C (Section 4.A).

6. Transfer 25µl of culture medium or buffer from each well to a 96-well opaque **white** luminometer plate at room temperature, and add 25µl of Luciferin Detection Reagent to initiate a luminescent reaction (for single-tube luminometers use appropriate luminometer tubes or cuvettes).
7. Incubate the plate at room temperature for 20 minutes, then read luminescence using a luminometer or CCD camera.

   Note: Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Do not use a fluorometer. Do not use filters with the luminometer.
8. Calculate net signals by subtracting background luminescence values (no-cell control) from test compound-treated and untreated (vehicle control) values.
9. Calculate percent change by dividing net treated values by net untreated values and multiplying by 100.
10. **Optional:** Perform the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570, G7571, G7572 and G7573) to normalize P450-Glo™ Assay values to cell number; see Section 11.D.

Table 6. P450-Glo™ Assay Setup.

			Medium Volume Required for Various Plate Sizes				
Assay	**Substrate**	**Final Substrate Concentration**	**96-Well Plate**	**48-Well Plate**	**24-Well Plate**	**6-Well Plate**	**Incubation Time**
CYP3A4	Luciferin-IPA	3µM (1:1,000 dilution*)	50µl	150µl	300µl	1.0ml	30–60 minutes
CYP3A4	Luciferin-PFBE	50µM (1:40 dilution*)	50µl	150µl	300µl	1.0ml	3–4 hours
CYP2C9	Luciferin-H	100µM (1:50 dilution*)	50µl	150µl	300µl	1.0ml	3–4 hours
CYP1A1 or CYP1B1	Luciferin-CEE	100µM (1:50 dilution*)	50µl	150µl	300µl	1.0ml	3 hours
CYP1A2	Luciferin-1A2	6µM (1:1,000 dilution**)	50µl	150µl	300µl	1.0ml	30–60 minutes

*Dilute the provided substrate stock solution in culture medium.
**Dilute Luciferin-1A2 stock solution in the Krebs-Henseleit Buffer or PBS containing 3mM salicylamide.

10.C. Lytic P450-Glo™ Assays Using Cultured Cells in Monolayers

Note: If you intend to read luminescence directly from culture wells, cells should be cultured on white-walled, collagen-coated culture plates with clear bottoms (e.g., BD BioCoat™ plates, BD Biosciences Cat.# 354650).

1. Treat cells with test compounds. For CYP gene induction studies, cells are typically treated with inducers for 24–72 hours. Optimal treatment time should be determined empirically; however, 48 hours is a common starting point. See Table 7 for examples of CYP gene inducers that can be used as positive controls. Change the medium with test compounds, etc., once daily for the duration of the treatment time.

2. For **CYP3A4/Luciferin-IPA**, **CYP3A4/Luciferin-PFBE, CYP2C9/Luciferin-H**, **CYP1A1/Luciferin-CEE**, and **CYP1B1/Luciferin-CEE** assays, replace culture medium with fresh medium containing a luminogenic CYP substrate after the experimental treatment as indicated in Table 6. See Section 4.B for substrate preparation instructions.

   Optional: Wash cells with medium or phosphate buffered saline before adding medium with substrate. Some compounds that induce CYP gene expression also inhibit the CYP enzyme activity that has been induced. To observe induction, you may need to remove the inducer by including a wash step prior to adding a luminogenic substrate.

   For **CYP1A2/Luciferin-1A2** assays, remove the culture medium completely after treatment and wash cells twice with two volumes of Krebs-Henseleit buffer or PBS. Add diluted Luciferin-1A2 in Krebs-Henseleit Buffer (e.g., Sigma Cat.# K3753) or phosphate buffered saline (PBS). This P450-Glo™ Assay cannot be performed in most cell culture media because they contain L-cysteine, which interferes with the CYP1A2/Luciferin-1A2 assay.

   Notes:

   1. See Section 4.B for substrate preparation instructions.
   2. If inhibitors of basal or induced CYP enzyme activity are being tested, add them at this point with the luminogenic substrate.
   3. Salicylamide is added to inhibit phase II conjugation of the CYP product. Prepare a fresh 3M salicylamide stock in DMSO from the powder every time. When diluting salicylamide in Krebs-Henseleit Buffer, a white "precipitate" of salicylamide may occur. With mixing, all of the salicylamide will dissolve in the Krebs-Henseleit Buffer or PBS.
   4. To determine background luminescence, add luminogenic substrate in medium to a set of empty wells (no cells). For the CYP1A2/Luciferin-1A2 assay, add Luciferin-1A2 in the Krebs-Henseleit Buffer containing 3mM salicylamide to a set of empty wells (no cells).

3. To determine background luminescence, add luminogenic substrate in medium to a set of empty wells (no cells). For CYP1A2/Luciferin-1A2 assays, add Luciferin-1A2 in the Krebs-Heneseleit Buffer of PBS containing 3mM salicylamide to a set of empty wells (no cells).

4. Incubate plates at 37°C, 5% CO_2 for 30–60 minutes with Luciferin-IPA and Luciferin-1A2 for 3–4 hours for all other luminogenic substrate as indicated in Table 6.

5. During the incubation, prepare the Luciferin Detection Reagent by transferring the entire contents of the bottle of the appropriate reconstitution buffer to the amber bottle containing Luciferin Detection Reagent. For the CYP1A2/Luciferin-1A2 assay, add the supplied D-cysteine, 500X, to the reconstituted Luciferin Detection Reagent to a final concentration of 1X. For 10ml (Part# V859A and V865A) and 50ml (Part# V859B and V865B) reconstituted Luciferin Detection Reagent, add 20µl and 100µl of the supplied D-cysteine, 500X, resepectively. Mix by swirling or inverting several times, and equilibrate to room temperature before use. Store the unused portion at –20°C (Section 4.A).

6. Add an equal volume of Luciferin Detection Reagent to each well, and mix briefly on a multiwell plate shaker or by gently tapping or swirling the plate to form a lysate. .

7. **Option 1:** Transfer 50µl of lysate from each well to a 96-well opaque **white** luminometer plate at room temperature (for single-tube luminometers use appropriate luminometer tubes or cuvettes).

   Option 2: Read luminescence directly from cell culture plate. In this case, to avoid luminescent crosstalk between wells, cells must be grown in a white-walled culture plate with clear-bottom wells.

8. Equilibrate the plate at room temperature for 15–20 minutes.

   Note: Luminescence from lysates of some, but not all, cell types may decay rapidly, so it might be necessary to read luminescence as soon as possible after adding Luciferin Detection Reagent.

9. Read luminescence using a luminometer or CCD camera.

   Note: Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Do not use a fluorometer. Do not use filters with the luminometer.

10. Calculate net signals by subtracting background luminescence values (no-cell control) from test compound-treated and untreated (vehicle control) values.

11. Calculate percent change by dividing net treated values by net untreated values and multiplying by 100.

10.D. Lytic P450-Glo™ Assays with Suspension Cells

We recommend this protocol for monitoring basal CYP activity and inhibition of basal activity using Luciferin-IPA for CYP3A4 and Luciferin-H for CYP2C9. We do not recommend the use of suspension cells to measure CYP induction.

This protocol is written for 96-well plates. For smaller well formats, scale volumes as necessary.

Use 96-well plates with white walls.

Follow the vendor's directions for thawing and counting cryopreserved hepatocytes, or use fresh hepatocytes in suspension.

1. Prepare a 2X P450-Glo™ substrate/2X test compound solution in culture medium. Prepare 50µl for each well. The recommended substrate concentrations are showing in Table 6.
2. Adjust cell concentration to 2 x 10^6 cells/ml in vendor-recommended serum-free medium.
3. Add 50µl of homogeneous cell suspension to each well.
4. Add 50µl of 2X P450-Glo™ substrate/2X test compound solution to the cell suspension in each well. For control wells without cells, add 50µl of 2X P450-Glo™ substrate to 50µl of culture medium.
5. Incubate the plate at 37°C, 5% CO_2 for 15 minutes for Luciferin-IPA reactions or 2 hours for Luciferin-H reactions.
6. During the incubation, prepare the Luciferin Detection Reagent by transferring the entire contents of the bottle of the appropriate reconstitution buffer to the amber bottle containing Luciferin Detection Reagent. For the CYP1A2/Luciferin-1A2 assay, add the supplied D-cysteine, 500X, to the reconstituted Luciferin Detection Reagent to a final concentration of 1X. For 10ml (Part# V859A and V865A) and 50ml (Part# V859B and V865B) reconstituted Luciferin Detection Reagent, add 20µl and 100µl of the supplied D-cysteine, 500X, resepectively. Mix by swirling or inverting several times, and equilibrate to room temperature before use. Store the unused portion at –20°C (Section 4.A).
6. Add an equal volume (100µl) of Luciferin Detection Reagent to each well. Shake briefly on orbital shaker.
7. Equilibrate the plate at room temperature for 15–20 minutes.

   Note: Luminescence from lysates of some, but not all, cell types may decay rapidly, so it might be necessary to read luminescence as soon as possible after adding Luciferin Detection Reagent.

8. Read luminescence using a luminometer or CCD camera.

   Note: Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Do not use a fluorometer. Do not use filters with the luminometer.

9. Calculate net signals by subtracting background luminescence values (no-cell control) from test compound-treated and untreated (vehicle control) cells.

10. Calculate percent change by dividing net treated values by net untreated values and multiplying by 100.

11. General Considerations for Cell-Based Assays

11.A. Examples of CYP Gene Inducers

Upregulation of CYP genes occurs when a test compound binds one of several nuclear receptors and then activates transcription of a CYP gene (9). The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) regulate the CYP2C8, CYP2C9, CYP2C19 and CYP3A4 genes (9–11); the glucocorticoid receptor (GR) regulates CYP3A4 genes; the aryl hydrocarbon receptor (AHR) regulates CYP1A and CYP1B1 (12) genes.

The compounds listed in Table 7 are well known CYP gene inducers. These can be included as positive controls for induction when screening test compounds with unknown effects on CYP gene expression.

Table 7. Common CYP Gene Inducers.

CYP Gene	Inducers		Pathway
CYP3A4	25µM	rifampicin	PXR
	500µM	phenobarbitol	CAR
	50µM	dexamethasone	GR
CYP2C9	25µM	rifampicin	PXR/CAR
	500µM	phenobarbitol	
CYP1A1/1A2/1B1	10nM	2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD)	AHR
	1µM	3-methylcholanthrene	
	100µM	omeprazole	

11.B. Fresh Hepatocyte Cell Cultures

Fresh human hepatocytes adherent on collagen-coated plates or in suspension can be obtained for overnight delivery from various vendors (e.g., Celsis/In Vitro Technologies, Inc.). The following considerations for adherent hepatocyte culture conditions have been used successfully with the P450-Glo™ Assay.

Note: The hepatocyte phenotype is best maintained when cells are near confluence.

1. On day 1, viable hepatocytes from a vendor arrive in a multiwell plate at ambient temperature. Remove adhesive seal from the plate, and replace with a loose-fitting multiwell plate lid. Incubate the plate for 2 hours in a 5% CO_2, 37°C incubator in the shipping medium.

11.B. Fresh Hepatocyte Cell Cultures (continued)

2. After the 2-hour incubation, remove the shipping medium, and replace with 37°C culture medium formulated for hepatocytes (e.g., In Vitro GRO Hepatocyte Incubation Medium, Celsis/In Vitro Technologies Inc.).
3. Return the plates to the incubator for 1 hour if you are using the sandwich culture method (Step 4). Otherwise, incubate overnight in the incubator.
4. **Sandwich culture method (optional):** After the 1-hour incubation, add the same volume of cold medium (approximately 4°C) containing 0.25mg/ml Matrigel™ (BD Biosciences) to each well. Return the plates to the incubator overnight.
5. On day two, remove the culture medium, and replace with fresh serum-free medium (without Matrigel™) that contains a control inducer, test compound or vehicle alone.
6. Change the medium with test compounds or controls once daily for the duration of the experiment. For CYP gene induction studies, cells are typically exposed for 24–72 hours. Optimal length of treatment time should be determined empirically, though maximal inductions are typically reached after 48 hours of exposure to an inducer.
7. Perform the P450-Glo™ Assay as described in Section 10.B, 10.C or 10.D.

11.C. Cryopreserved Hepatocytes

Cryopreserved primary human and animal hepatocytes are available from several suppliers (e.g., Celsis/In Vitro Technologies, Inc.). Store the cells in liquid nitrogen. Note that some cryopreserved hepatocytes may not adhere well to 96-well plates and may require wells with a larger surface area. For optimal results, use cells designated as "platable" (i.e., cells adhere and form monolayers in culture) by the supplier.

1. On day 1, thaw the cells as recommended by the supplier. This typically involves a specialized isolation procedure or thawing medium (e.g., InVitroGro Hepatocyte Thawing Medium, Celsis/In Vitro Technologies, Inc.; Hepatocyte Isolation Kit, XenoTech, LLC).
2. Estimate the percentage of live cells (e.g., by trypan blue exclusion).
3. Seed approximately 1.5×10^5 cells per cm^2 on collagen-coated tissue culture plates in a hepatocytes culture medium (e.g., In Vitro GRO Hepatocyte Incubation Medium, Celsis/In Vitro Technologies Inc.).
4. Incubate cells for 6 hours if you are using the sandwich culture method (Step 5). Otherwise, incubate overnight.
5. **Sandwich culture method (optional):** After the 6-hour incubation, add the same volume of cold medium (approximately 4°C) containing 0.25mg/ml Matrigel™ (BD Biosciences) to each well. Return the plates to the incubator overnight.

6. On day two, remove the culture medium, and replace with fresh serum-free medium (without Matrigel™) that contains a control inducer, test compound or vehicle alone.

7. Change the medium with test compounds or controls once daily for the duration of the experiment. For CYP gene induction studies, cells are typically exposed for 24–72 hours days. Optimal length of treatment time should be determined empirically, though maximal inductions are typically reached after 48 hours of exposure to an inducer.

8. Perform the P450-Glo™ Assay as described in Section 10.B, 10.C or 10.D.

11.D. Normalizing to Viable Cell Number

After sampling reaction for the nonlytic P450-Glo™ Assay (Section 10.B), the cells remain intact. We recommend performing a CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570, G7571, G7572, G7573) for each culture well to estimate cell number. Results then can be normalized to cell number by dividing the P450-Glo™ Assay values by the CellTiter-Glo® Assay values. This compensates for variability in cell number due to inconsistent plating efficiency, toxicity or proliferative effects of certain test compounds.

For information about the CellTiter-Glo® Assay, refer to Technical Bulletin #TB288 while considering the following examples:

- When using the CellTiter-Glo® Luminescent Cell Viability Assay, avoid cross-contamination of the P450-Glo™ reactions. The CellTiter-Glo® Reagent contains luciferin, which will interfere with the P450-Glo™ Assay. Prepare the CellTiter-Glo® Reagent away from the area where the P450-Glo™ reaction will be performed. If contamination does occur, wash the area well with dilute detergent and alcohol. Use aerosol-resistant tips.

- After Step 6 in Section 10.B (Nonlytic P450-Glo™ Assays Using Cultured Cells in Monolayers), there is 25µl of medium remaining in the P450-Glo™ Assay wells. Add an equal volume (25µl) of the CellTiter-Glo® Reagent to those wells. After shaking for a few minutes, remove an aliquot (such as 40µl) to an opaque white luminometer plate to measure luminescence. Alternatively, if cells are cultured in white-walled plates with clear bottoms, luminescence can be measured directly from the culture plate without transfer.

12. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
High background luminescence	Luciferin contamination in one or more of the reaction components. • Avoid workspaces and pipettes that are used with luciferin-containing solutions, including luminescence-based cell viability, apoptosis or gene reporter assays. • Decontaminate work surfaces by wiping with a detergent solution or ethanol and rinsing with clean water. Rinse pipettes and other labware with distilled water multiple times. For automated dispensing systems, replace any components that have been used to dispense luciferin-containing solutions.
	Contamination of minus-P450 control reactions with a CYP isoform that reacts with the luminogenic substrate of interest. • Choose a control preparation known to be free of CYP activity. • Avoid contact between the inactive control and active preparations of CYP.
	Substrate was stored improperly. Store Luciferin-PPXE at or below –70°C, protected from light; store all other P450-Glo™ substrates at –20°C, protected from light.
Low luminescent signal	Use only white opaque luminometer plates. Do not use black plates or clear plates. Best results are obtained with nontreated, white, polystyrene plates (e.g., Costar® 96-well plates, Cat.# 3912, or white 96 MicroWell® plates, Nunc Cat.# 236108). CYP activity may be inhibited nonspecifically by binding of CYP membranes or substrates to a surface that has been treated to enhance hydrophobicity. Reactions with Luciferin-IPA are sensitive to this effect.

Symptoms	Causes and Comments
Low luminescent signal (continued)	Low CYP activity in enzyme preparation. • Store the CYP preparations at –70°C. Storing CYP enzymes at –20°C leads to a loss of activity. Dispense the membranes into single-use aliquots to avoid multiple freeze-thaw cycles. • Thaw the CYP preparation immediately before use. Extended incubations on ice or at room temperature may lead to enzyme inactivation. • For recombinant CYP2C8, 2C9, 2C19, 3A4, 3A5 and 3A7, use enzyme preparations that are supplemented with CYP450 reductase and cytochrome b5. • For recombinant CYP2B6 reactions with Luciferin-ME EGE, use an enzyme preparation without added cytochrome b5.
	Use Figure 8 to determine whether brighter signals are obtained at room temperature or 37°C for a given reaction time.
	The Luciferin Detection Reagent was reconstituted with the wrong buffer. For CYP1A1, 1A2, 1B1, 2C8, 2C9, 3A4/Luciferin-BE, 3A4/Luciferin-PFBE, 3A4/Luciferin-PPXE and 3A7 assays, use the supplied Reconstitution Buffer to reconstitute the lyophilized Luciferin Detection Reagent. For the CYP2C19, 2D6 and 3A4/Luciferin-IPA assays, use the supplied Reconstitution Buffer with esterase to reconstitute the lyophilized Luciferin Detection Reagent. These buffers are not interchangeable.
	The P450-Glo™ substrate formed a precipitate upon thawing or dilution in aqueous mixtures. • Briefly warm thawed solution to 37°C, then vortex to dissolve the substrate. • KPO_4 buffer was added directly to the 4X CYP3A4 reaction mixture. The 4X KPO_4 buffer concentration for CYP3A4 reactions with Luciferin-BE, Luciferin-PFBE and Luciferin-PPXE is 800mM, and this may cause the substrate to precipitate. By introducing KPO_4 buffer to the reaction as part of the NADPH regeneration mixture, the substrate is not exposed to the high KPO_4 buffer concentration, and precipitation is avoided.

12. Troubleshooting (continued)

Symptoms	Causes and Comments
Low luminescent signal from CYP1A2/Luciferin-1A2 assay	The Luciferin Detection Reagent was not supplemented with D-Cysteine. D-Cysteine is required to convert the product of CYP1A2/ Luciferin-1A2 reactions into D-luciferin.
Unexpected inhibition of P450-Glo™ Assay by test compound	Luciferase or esterase inhibition. Screen compounds using multiple CYP enzymes. Inhibition of only a subset of enzymes indicates that the test compound is not a luciferase inhibitor.
	Luciferase or esterase inhibition. Luciferase is used to generate luminescence in the P450-Glo™ Assays. A mixture of porcine esterases is used in the CYP2C19, 2D6 and 3A4/Luciferin-IPA Luciferin Detection Reagent to process the product of their respective reactions. The potential for inhibition of luciferase or esterase has been minimized by using an engineered luciferase, maintaining high enzyme concentrations and using reaction chemistries that reduce the effects of potential inhibitors. For example, 10µM resveratrol inhibits wildtype firefly luciferase (13) but does not inhibit the luciferase used in the P450-Glo™ Assay. Also, 10µM of the esterase competitive inhibitors ethyl butyrate, ethyl acetate and 4-nitrophenyl acetate had little or no effect on assay signal (98.5% ± 2.1%, 98.8% ± 1.1% and 98.4% ± 1.6% of control reactions, respectively). To test for luciferase inhibition, assemble two reactions, one with equal volumes of reconstituted Luciferin Detection Reagent and 400nM Beetle Luciferin, Potassium Salt (Cat.# E1601), and a second reaction with equal volumes of reconstituted Luciferin Detection Reagent and 400nM beetle luciferin plus the test compound. Incubate reactions for 10 minutes at room temperature, then measure the luminescence. A decrease in luminescence in the presence of the test compound indicates luciferase inhibition.

Symptoms	Causes and Comments
Unexpected inhibition of P450-Glo™ Assay by test compound (continued)	If luciferase inhibition has been ruled out as a possible cause, perform the following test for esterase inhibition (CYP2C19, 2D6 and 3A4/Luciferin-IPA assays only). Perform CYP2C19, 2D6 or 3A4/Luciferin-IPA reactions without test compound. Add Luciferin Detection Reagent to a control reaction and Luciferin Detection Reagent plus the test compound to a test reaction. Diminished signal in the test reaction indicates esterase inhibition.
	Inhibition of the NADPH regeneration system. Concerns that test compounds may inhibit the NADPH regeneration system and cause an apparent inhibition of CYP activity are unwarranted. The system generates an excess of NADPH, which remains at a nonlimiting concentration over the course of a reaction even in the absence of continual synthesis.
	Inhibition of CYP by an organic solvent. Minimize solvent concentration, or use a different solvent for the test compound. DMSO is a known CYP3A4 inhibitor (2).
Luciferin-PPXE substrate formed precipitate in the 4X Luciferin-PPXE/CYP3A4 reaction mixture	Use 100mM Tris-HCl (pH 7.5), not water, to prepare the 4X Luciferin-PPXE/CYP3A4 reaction mixture. Luciferin-PPXE has greater solubility in Tris-HCl.
	When preparing the 4X Luciferin-PPXE/CYP3A4 4X reaction mixture, mix the Luciferin-PPXE and Tris-HCl (pH 7.5) immediately upon combining.

12. Troubleshooting (continued)

Symptoms	Causes and Comments
P450 induction expected but not observed in a cell-based assay	Cell culture conditions did not support maintenance of the inducible phenotype. See Sections 11.B and 11.C.
	The CYP gene inducer is also an inhibitor of the target CYP enzyme. Induction may have occurred at the transcriptional level, resulting in an increase in the amount of CYP present, but an increase in activity is not observed because the gene inducer inhibits the enzyme activity. For reversible inhibitors, relieve enzyme inhibition by performing a wash step to remove the inducer before adding the P450-Glo™ substrate.
	The amount and extent of CYP enzyme induction in human hepatocytes is donor-dependent. Some individuals may exhibit poor P450 induction. Other individuals may show unexpected P450 induction.
Low luminescent signal from cell-based assays with Luciferin-1A2	The Luciferin Detection Reagent was not supplemented with D-Cysteine. D-Cysteine is required to convert the product of CYP1A2/ Luciferin-1A2 reactions into D-luciferin.
	The P450-Glo™ Assay was performed in cell culture medium, not in Krebs-Henseleit Buffer. The L-cysteine in the cell culture medium interferes with the Luciferin-1A2 substrate in the P450-Glo™ Assay.
	Salicylamide was not added to the Luciferin-1A2 substrate solution in Krebs-Henseleit Buffer. Salicylamide inhibits phase II conjugation reaction of the P450 product.
High luminescent signal in random wells of the plate	Possible luciferin contamination. Avoid luminometers that are used with luciferin-containing solutions, including luminescence-based cell viability, apoptosis or gene reporter assays.

13. Appendix

13.A. Composition of Buffers and Solutions

1M potassium phosphate buffer (pH 7.4)

13.94g	potassium phosphate dibasic, anhydrous
2.72g	potassium phosphate monobasic, anhydrous

Bring the volume to approximately 90ml with deionized water. Adjust to pH 7.4 with KOH or H_3PO_4. Add deionized water to a final volume of 100ml.

Solution A, NADPH Regeneration System (20X concentration)

26mM	NADP+
66mM	glucose-6-phosphate
66mM	$MgCl_2$

Solution B, NADPH Regeneration System (100X concentration)

40U/ml	glucose-6-phosphate dehydrogenase in 5mM sodium citrate (pH 5.5)

13.B. Reagent Suppliers

Promega offers the P450-Glo™ Screening Systems, which include recombinant CYP enzymes and control membranes devoid of CYP activity. P450-Glo™ Screening Systems are available for CYP1A2, 2C9, 2C19, 2D6 and 3A4 (Section 13.D).

The NADPH Regeneration System (Cat.# V9510) is not supplied with the P450-Glo™ Assays and must be purchased separately. See below for more information. The NADPH Regeneration System is **not required** for cell-based assays. In cell-based assays, NADPH in the cell is sufficient to support CYP activity.

Active Cytochrome P450 Preparations

Active CYP preparations include recombinant CYP1A1, 1A2, 1B1, 2C8, 2C9, 2C19, 2D6, 3A4, 3A7 and human liver microsomes. The CYP preparations should contain enough CYP450 reductase to support CYP activity. Some preparations also may contain cytochrome b5 for enhanced activity. Most recombinant CYP preparations are not limited for CYP450 reductase because they are prepared from a heterologous expression system with co-expression of CYP and CYP450 reductase. Control membrane preparations are typically made from cells of the heterologous expression system but without recombinant CYP expression. Insect cells or *E. coli* expression systems are most commonly used. Native levels of CYP450 reductase present in liver microsomes are sufficient to support cytochrome P450 activity. Suppliers of active CYP preparations are listed below.

Discovery Labware, Inc./BD Gentest
Life Technologies
Cypex
Oxford Biomedical Research
Moltox, Inc.
Sigma-Aldrich Co.
Xenotech, LLC
New England Biolabs
Celsis/In Vitro Technologies

13.B. Reagent Suppliers (continued)

NADPH Regeneration System

The NADPH regeneration system reduces NADP+ to NADPH. The NADPH Regeneration System available from Promega (Cat.# V9510) consists of two reagents, Solution A and Solution B. Solution A contains the substrates NADP+ and glucose-6-phosphate and is supplied at a 20X concentration. Solution B contains the enzyme glucose-6-phosphate dehydrogenase at a 100X concentration. The two solutions are combined before use at a 2X concentration, and the NADPH generated serves as the source of electrons for the CYP oxidative reactions. When Solution A and Solution B are combined, reduction of NADP+ to NADPH occurs rapidly. Within 5–10 minutes at room temperature, the regeneration system is fully charged. The P450-Glo™ Assays are initiated by adding the 2X NADPH regeneration system to the CYP assays.

The 2X NADPH regeneration system for use with all P450-Glo™ Assays except CYP3A4 with Luciferin-BE, Luciferin-PFBE or Luciferin-PPXE contains 2.6mM NADP+, 6.6mM glucose-6-phosphate, 6.6mM $MgCl_2$ and 0.8U/ml glucose-6-phosphate dehydrogenase. The 2X NADPH regeneration system for CYP3A4 with Luciferin-BE, Luciferin-PFBE or Luciferin-PPXE contains 2.6mM NADP+, 6.6mM glucose-6-phosphate, 6.6mM $MgCl_2$, 0.8U/ml glucose-6-phosphate dehydrogenase and 400mM KPO_4 buffer. A typical 50µl CYP assay will contain 2.5µl of Solution A and 0.5µl of Solution B.

Purified NADPH can be substituted for the 2X NADPH regeneration system in P450-Glo™ Assays. The final concentration in the CYP assay should be 100µM. NADPH can be purchased from Sigma-Aldrich and other chemical suppliers.

The NADPH Regeneration System is not required for cell-based assays. In cell-based assays, NADPH in the cell is sufficient to support CYP activity.

Stability

Solution A and B are stable for up to five freeze-thaw cycles. Both solutions may be held at room temperature for up to 2 hours without significant loss in the ability to generate NADPH. When combined, the resulting NADPH regeneration system remains charged at room temperature for up to 2 hours and is stable for repeated freeze-thaw cycles.

13.C. References

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13.D. Related Products

Product	Size	Cat.#
NADPH Regeneration System	1,000 assays	V9510
Beetle Luciferin, Potassium Salt	5mg	E1601
	50mg	E1602
	250mg	E1603
	1g	E1605
P450-Glo™ CYP1A2 Screening System	1,000 assays	V9770
P450-Glo™ CYP2C9 Screening System	1,000 assays	V9790
P450-Glo™ CYP2C19 Screening System	1,000 assays	V9880
P450-Glo™ CYP2D6 Screening System	1,000 assays	V9890
P450-Glo™ CYP3A4 Screening System	1,000 assays	V9800
P450-Glo™ CYP3A4 Screening System (Luciferin-PPXE) DMSO Tolerant Assay	1,000 assays	V9910
P450-Glo™ CYP3A4 Screening System with Luciferin-IPA	1,000 assays	V9920

Luminogenic Enzyme Substrates

Product	Size	Cat.#
Luciferin-NAT2	3mg	P1721
Luciferin-3A7	3mg	P1741
Luciferin-4A	3mg	P1621
Luciferin-4F2/3	3mg	P1651
Luciferin-4F12	3mg	P1661
Luciferin-2J2/4F12 (ester)	3mg	P1671
Luciferin-MultiCYP (ester)	3mg	P1731

Luciferin Detection Reagents

Product	Size	Cat.#
Luciferin Detection Reagent	50ml	V8921
Luciferase Detection Reagent with esterase	50ml	V8931

Additional sizes available. **Note:** Use Cat.# V8921 with Cat.# P1621, P1651, P1661, P1721, and P1741. Use Cat.# V8931 with Cat.# P1671 and P1731.

Monoamine Oxidase Assay

Product	Size	Cat.#
MAO-Glo™ Assay	200 assays	V1401
	1,000 assays	V1402

UGT Activity Assays

Product	Size	Cat.#
UGT-Glo™ Assay	200 assays	V2081
	1,000 assays	V2082
UGT-Glo™ UGT1A1 Screening System	200 assays	V2120
	1,000 assays	V2121
UGT-Glo™ UGT2B7 Screening System	200 assays	V2130
	1,000 assays	V2131

P-glycoprotein Assays

Product	Size	Cat.#
Pgp-Glo™ Assay System	10ml	V3591
Pgp-Glo™ Assay System with P-glycoprotein	10ml	V3601

Glutathione-S-Transferase Assay

Product	Size	Cat.#
GSH-Glo™ Glutathione Assay	10ml	V6911
	50ml	V6912

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay (ATP)	10 × 10ml	G7571
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS)	5,000 assays	G3581
CellTiter-Blue® Cell Viability Assay (Resazurin)	10 × 100ml	G8082
MultiTox-Fluor Multiplex Cytotoxicity Assay	2 × 50ml	G9202
CellTiter-Fluor™ Cell Viability Assay	2 × 50ml	G6082

Many of these products are available in additional sizes.

Apoptosis Assays

Product	Size	Cat.#
Apo-ONE® Homogeneous Caspase-3/7 Assay	100ml	G7791
Caspase-Glo® 3/7 Assay	100ml	G8092
Caspase-Glo® 8 Assay	100ml	G8202
Caspase-Glo® 9 Assay	100ml	G8212
Caspase-Glo® 6 Assay	50ml	G0971
Caspase-Glo® 2 Assay	50ml	G0941

All of these products are available in additional sizes.

Luminometers

Product	Size	Cat.#
GloMax®-Multi Base Instrument*	1 each	E7031
GloMax®-Multi Luminescence Module	1 each	E7041
GloMax®-Multi Fluorescence Module	1 each	E7051
GloMax®-Multi Absorbance Module	1 each	E7061
GloMax® 96 Microplate Luminometer	1 each	E6501
GloMax® 20/20 Luminometer	1 each	E5311

*Cat.# E7031 must be purchased with at least one detection module (Cat.# E7041, E7051, E7061).

[a]U.S. Pat. Nos. 6,602,677, 7,241,584, 8,030,017, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents pending.

[b]U.S. Pat. No. 7,692,022 and other patents pending.

[c]Patent Pending.

[d]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

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Promega Corporation · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA · Toll Free in USA 800-356-9526 · 608-274-4330 · Fax 608-277-2516
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