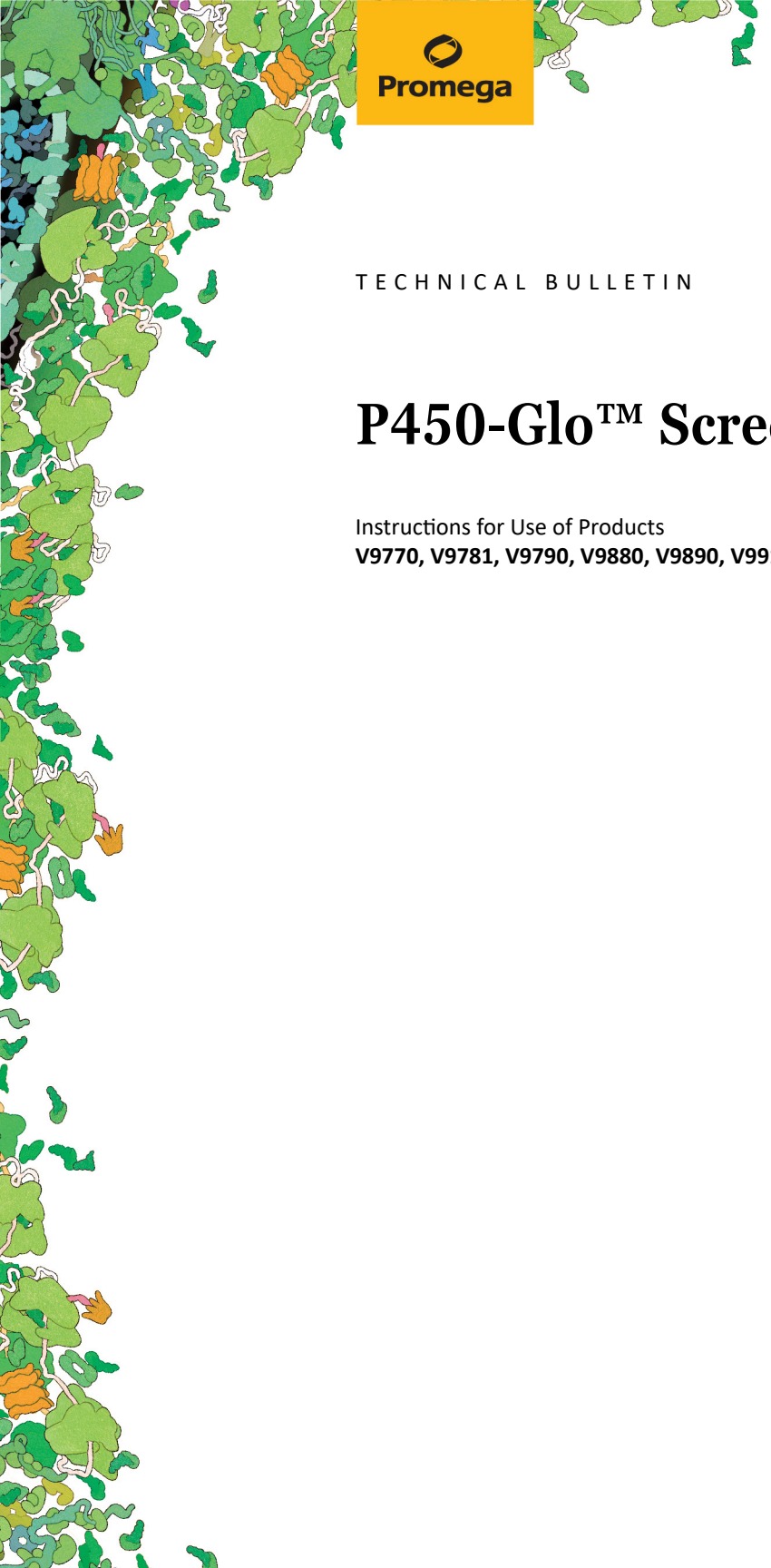


TECHNICAL BULLETIN

P450-Glo™ Screening Systems

Instructions for Use of Products
V9770, V9781, V9790, V9880, V9890, V9910 and V9920

Revised 3/22
TB340

Promega

P450-Glo™ Screening Systems

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

1. Description

The P450-Glo™ Screening Systems[a–f] provide a complete set of reagents for performing luminescent cytochrome P450 assays (1–3). The systems include a membrane preparation containing recombinant human cytochrome P450 (CYP) enzyme, negative control membranes, a luminogenic substrate appropriate for the CYP enzyme, NADPH regeneration system, reaction buffer, Luciferin Detection Reagent and Luciferin-Free Water. The membranes are prepared from baculovirus-infected insect cells and contain human CYP enzyme and P450 reductase (and cytochrome b5 for CYP2B6, 2C9, 2C19 and 3A4). The negative control membranes are devoid of CYP activity. The assays are ideal for testing the effects of drugs and new chemical entities on CYP enzyme activities.

The CYP reaction is performed by incubating a luminogenic CYP substrate with a CYP enzyme and NADPH regeneration system. The luminogenic P450-Glo™ substrates are derivatives of beetle luciferin [(4S)-4,5-dihydro-2-(6-hydroxybenzothiazolyl)-4-thiazolecarboxylic acid or D-luciferin], a substrate of firefly luciferase. The P450-Glo™

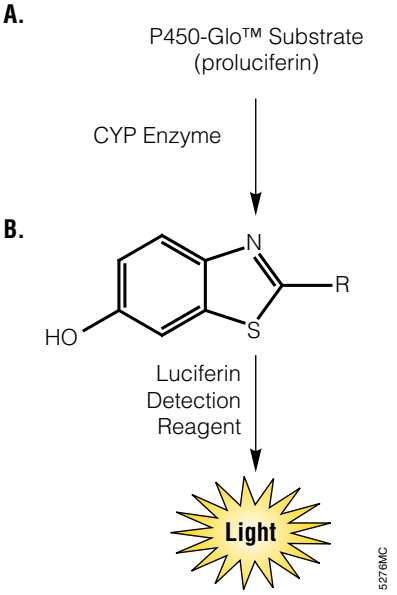


Figure 1. Conversion of P450-Glo™ substrate by cytochrome P450. CYP enzymes act on a luminogenic P450-Glo™ substrate (Reaction A) to produce a luciferin product that generates light with the Luciferin Detection Reagent (Reaction B), which is added after the CYP reaction has been completed. CYP selectivity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

substrates (Luciferin-2B6, Luciferin-IPA, Luciferin-ME, Luciferin-H, Luciferin-ME EGE, Luciferin-H EGE and Luciferin-PPXE) do not react with luciferase but are converted by CYP enzymes to a luciferin product that in turn reacts with a Luciferin Detection Reagent to produce light (Figure 1). Light is used to monitor CYP activity because the amount of light produced is proportional to the amount of luciferin product formed after the CYP reaction.

The CYP reactions are performed first, and then the reconstituted Luciferin Detection Reagent is added (Figure 2). This reagent simultaneously stops the CYP reaction and initiates a glow-type luminescent signal with a half-life greater than 2 hours. The glow-type luciferase reaction produces a stable signal that eliminates the need for strictly timed luminescence detection. Protocols are configured for multiwell plate formats but can be adapted easily for single-tube applications.

P450-Glo™ substrate selectivities of CYP enzymes are shown in Table 1.

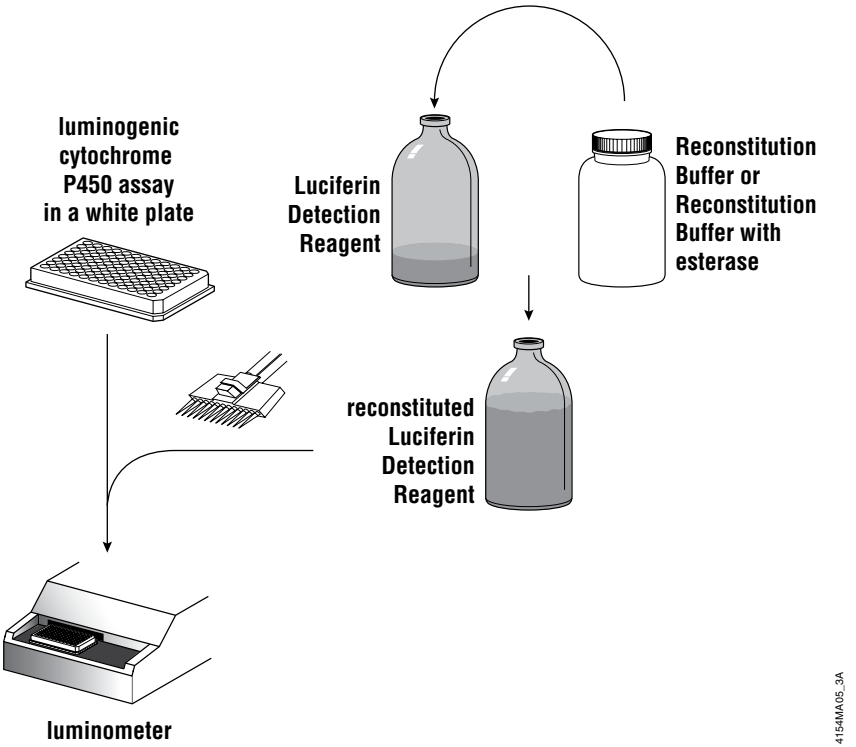
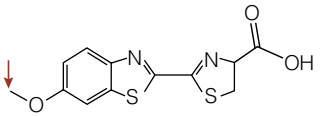
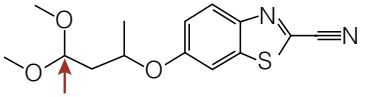
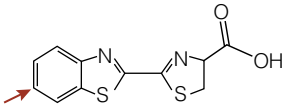
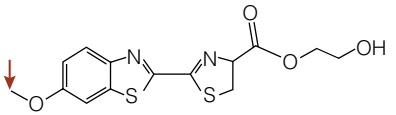
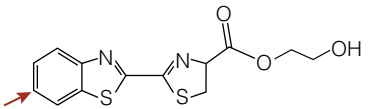
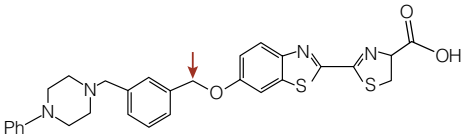
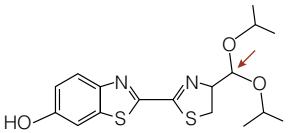


Figure 2. Flow diagram showing preparation and use of the reconstituted Luciferin Detection Reagent. For CYP1A2, 2B6, 2C9 and 3A4/Luciferin-PPXE assays, use the Reconstitution Buffer to reconstitute the lyophilized Luciferin Detection Reagent (for CYP2B6 assay, also add D-cysteine to 1X by dilution of the 500X stock solution). For CYP3A4/Luciferin-IPA, CYP2C19 and 2D6 Assays, use the Reconstitution Buffer with esterase to reconstitute the lyophilized Luciferin Detection Reagent. **Label the blank space on the Luciferin Detection Reagent label with the assay name to ensure the correct Luciferin Detection Reagent is used.**

1. Description (continued)

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

Substrate[1]	CYP Enzyme
 Luciferin-ME	CYP1A2
 Luciferin-2B6	CYP2B6[2]
 Luciferin-H	CYP2C9
 Luciferin-ME EGE	CYP2D6
 Luciferin-H EGE	CYP2C19
 Luciferin-PPXE	CYP3A4[3]
 Luciferin-IPA	CYP3A4[3]

Footnotes on following page.

[1]The arrow indicates the site of modification by CYP.
[2]The product of CYP2B6 and Luciferin-2B6 reaction is a luciferin precursor. D-luciferin is formed and detected by the Luciferin Detection Reagent supplemented with D-cysteine.
[3]CYP3A4 systems are available with two distinct CYP3A4 substrates: Luciferin-IPA or Luciferin-PPXE. See Table 1 notes for recommendations for choosing a CYP3A4 substrate.

Notes for Table 1

Two distinct substrates are available for the CYP3A4 enzyme.

Luciferin-IPA is the most sensitive and selective substrate for all CYP3A4 applications. The CYP3A4 reaction with Luciferin-IPA is only modestly inhibited by DMSO.

Luciferin-PPXE reactions for CYP3A4 are highly insensitive to DMSO, with little or no inhibition at or below 0.25% DMSO.

Advantages of the P450-Glo™ Screening System include:

Complete System: The protocol and reagents have been tested for optimal performance.

Speed: The luminescent format eliminates the need for time-consuming analyses such as mass spectrometry, HPLC or thin-layer chromatography.

Simplified Method: The simple "add and read" protocol makes the assay amenable to high-throughput screening in multiwell plates.

Greater Sensitivity: Less CYP is required in these assays than in typical HPLC or fluorometric methods due to the enhanced sensitivity.

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.

DMSO Tolerance: The P450-Glo™ reactions are not inhibited substantially by DMSO at concentrations typically encountered (e.g., ≤0.25%).

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.

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP1A2 Screening System	**1,000 assays**	**V9770**

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

Cat.# V8772, P450-Glo™ CYP1A2 Assay, which includes:
- 1ml Luciferin-ME, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 50ml Reconstitution Buffer

Cat.# V4770, Human CYP1A2 Enzyme System, which includes:
- 500µl CYP1A2 (1pmol/µl) + Reductase*
- 100µl Control Membranes*
- 5.0ml Potassium Phosphate Buffer, 1M (pH 7.4)
- 50ml Luciferin-Free Water
- 2.75ml Solution A, NADPH Regeneration System
- 0.6ml Solution B, NADPH Regeneration System

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP2B6 Screening System	**1,000 assays**	**V9781**

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

Cat.# V8322, P450-Glo™ CYP2B6 Assay, which includes:
- 60µl Luciferin-2B6, 3mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 50ml Reconstitution Buffer
- 100µl D-Cysteine Solution, 2M

Cat.# V7531, Human CYP2B6 Enzyme System, which includes:
- 100µl CYP2B6 (1pmol/µl) + Reductase + b5*
- 100µl Control Membranes*
- 5.0ml Potassium Phosphate Buffer, 1M (pH 7.4)
- 50ml Luciferin-Free Water
- 2.75ml Solution A, NADPH Regeneration System
- 0.6ml Solution B, NADPH Regeneration System

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP2C9 Screening System	**1,000 assays**	**V9790**

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

Cat.# V8792, P450-Glo™ CYP2C9 Assay, which includes:
- 1ml Luciferin-H, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 50ml Reconstitution Buffer

Cat.# V4790, Human CYP2C9 Enzyme System, which includes:
- 500µl CYP2C9 (1pmol/µl) + Reductase + b5*[1]
- 100µl Control Membranes*
- 5.0ml Potassium Phosphate Buffer, 1M (pH 7.4)
- 50ml Luciferin-Free Water
- 2.75ml Solution A, NADPH Regeneration System
- 0.6ml Solution B, NADPH Regeneration System

[1]Recombinant human CYP2C9*1 (Arg144) is expressed from a cDNA using a baculovirus expression system. CYP2C9*1 is the most common human CYP2C9 allele (4).

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP2C19 Screening System	**1,000 assays**	**V9880**

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

Cat.# V8882, P-Glo™ CYP2C19 Assay, which includes:
- 2 × 123µg Luciferin-H EGE
- 1 vial Luciferin Detection Reagent (lyophilized)
- 50ml Reconstitution Buffer with esterase

Cat.# V4880, Human CYP2C19 Enzyme System, which includes:
- 250µl CYP2C19 (1pmol/µl) + Reductase + b5*
- 100µl Control Membranes*
- 5.0ml Potassium Phosphate Buffer, 1M (pH 7.4)
- 50ml Luciferin-Free Water
- 2.75ml Solution A, NADPH Regeneration System
- 0.6ml Solution B, NADPH Regeneration System

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP2D6 Screening System	**1,000 assays**	**V9890**

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

Cat.# V8892, P450-Glo™ CYP2D6 Assay, which includes:
- 900µg Luciferin-ME EGE
- 1 vial Luciferin Detection Reagent (lyophilized)
- 50ml Reconstitution Buffer with esterase

Cat.# V4890, Human CYP2D6 Enzyme System, which includes:
- 250µl CYP2D6 (1pmol/µl) + Reductase*[1]
- 100µl Control Membranes*
- 5.0ml Potassium Phosphate Buffer, 1M (pH 7.4)
- 50ml Luciferin-Free Water
- 2.75ml Solution A, NADPH Regeneration System
- 0.6ml Solution B, NADPH Regeneration System

[1]Recombinant human CYP2D6*1 is expressed from a cDNA using a baculovirus expression system. CYP2D6*1 is the most common human CYP2D6 allele (5).

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP3A4 Screening System with Luciferin-IPA	**1,000 assays**	**V9920**

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

Cat.# V9002, P450-Glo™ CYP3A4 Assay, which includes:
- 60µl Luciferin-IPA, 3mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 50ml Reconstitution Buffer with esterase

Cat.# V4820, Human CYP3A4 Enzyme System, which includes:
- 100µl CYP3A4 (1pmol/µl) + Reductase + b5*
- 100µl Control Membranes*
- 5.0ml Potassium Phosphate Buffer, 1M (pH 7.4)
- 50ml Luciferin-Free Water
- 2.75ml Solution A, NADPH Regeneration System
- 0.6ml Solution B, NADPH Regeneration System

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP3A4 Screening System (Luciferin-PPXE) DMSO Tolerant Assay	**1,000 assays**	**V9910**

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

Cat.# V8912, P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO Tolerant Assay, which includes:

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

Cat.# V4910, Human CYP3A4 Enzyme System, which includes:

- 500µl CYP3A4 (1pmol/µl) + Reductase + b5*
- 100µl Control Membranes*
- 2 × 5.0ml Potassium Phosphate Buffer, 1M (pH 7.4)
- 50ml Luciferin-Free Water
- 2.75ml Solution A, NADPH Regeneration System
- 0.6ml Solution B, NADPH Regeneration System
- 1.0ml 2M Tris-HCl (pH 7.5), 20X

*CYP (1pmol/µl) + Reductase or CYP (1pmol/µl) + Reductase + b5 (referred to as CYP membranes in this Technical Bulletin) is a membrane preparation that contains recombinant human CYP expressed from a cDNA using a baculovirus expression system. These membranes also contain cDNA-expressed human P450 reductase or P450 reductase and cytochrome b5, and are prepared as a microsomal fraction from baculovirus-infected insect cells. The Control Membranes are prepared from wildtype baculovirus-infected insect cells and have a total protein concentration of 5mg/ml.

Storage Conditions: Store the CYP1A2, 2B6, 2C9, 2C19, 2D6, 3A4 and Control Membranes at –70°C. CYP enzymes may lose activity with repeated freeze-thaw cycles. Avoid multiple freeze-thaw cycles by dispensing the CYP1A2, 2B6, 2C9, 2C19, 2D6 and 3A4 membranes into single-use aliquots, and store at –70°C. Store other components at –20°C or –70°C, except Luciferin-PPXE, which must be stored at –70°C. Protect components from light.

The reconstituted Luciferin 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 all components.

3. Assay Conditions

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

Step 1. The Cytochrome P450 Reaction: The P450-Glo™ substrates are converted by CYP enzymes to a luciferin product.

a. Prepare a 4X cytochrome P450 reaction mixture with CYP enzyme, P450-Glo™ substrate and KPO_4 buffer as indicated in each protocol.

b. Add one volume of this mixture (e.g., 12.5µl in a 96-well plate) to an opaque white 96-well plate.

c. Add an equal volume of test compound solution to the 4X cytochrome P450 reaction mixture (e.g., 12.5µl added to bring the volume to 25µl in a 96-well plate).

d. Initiate reactions by adding 2X NADPH Regeneration System (Cat.# V9510) (e.g., 25µl added for a final volume of 50µl in a 96-well plate).

Table 2 lists the reaction components, recommended reagent concentrations and incubation times. Assay details are provided with the individual CYP protocols.

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. The P450-Glo™ Assays use a proprietary luciferase (Ultra-Glo™ Luciferase) to generate a stable glow-type luminescent signal. This eliminates the need for strictly timed luminescence detection.

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

Table 2. Recommended Final Concentrations of Reaction Components in the P450-Glo™ Assays.

Cytochrome P450	CYP per Reaction (96-Well Plate)[1]	Potassium Phosphate Concentration	Substrate Concentration (K_m concentration)	Incubation Time (37°C/RT)[2]
CYP1A2	0.5pmol (0.5µl)	100mM	100µM Luciferin-ME	10/30 minutes
CYP2B6	0.1pmol (0.1µl)	100mM	3µM Luciferin-2B6	10/10 minutes
CYP2C9	0.5pmol (0.5µl)	25mM	100µM Luciferin-H	30/30 minutes
CYP2C19	0.25pmol (0.25µl)	50mM	10µM Luciferin-H EGE	20/30 minutes
CYP2D6	0.25pmol (0.25µl)	100mM	30µM Luciferin-ME EGE	30/45 minutes
CYP3A4	0.5pmol (0.5µl)	200mM	25µM Luciferin-PPXE	15/30 minutes
CYP3A4	0.1pmol (0.1µl)	100mM	3µM Luciferin-IPA	10/10 minutes

[1]The recommended amount and volume of CYP per 50µl reaction given here are for a 96-well plate. For smaller well formats, scale volumes as necessary.

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

3.A. Cytochrome P450 Concentration

Although it is necessary to use enough enzyme to generate a detectable amount of D-luciferin, large amounts of protein or phospholipid from the microsome preparations can nonspecifically bind to a drug or inhibitor, leading to a reduction in the effective concentration and overestimation of K_m and K_i values (6). The amounts of CYP recommended in Table 2 give strong signals and are low enough so that nonspecific binding of CYP substrates was not detected (1). CYP concentrations can be increased for brighter signals or reduced further. Use the enzyme titration curves shown in Figure 3 as a guide if you prefer to use more or less enzyme.

3.B. Assay Time and Temperature

CYP reactions are generally performed at 37°C, but they also may be performed at room temperature (approximately 20–25°C). The suggested incubation times (Table 2) give strong signals and are within the linear range as shown in Figure 4. If you prefer a different incubation time, refer to Figure 4 to determine if a shorter time will produce adequate signal or if a longer time remains within the linear range.

3.B. Assay Time and Temperature

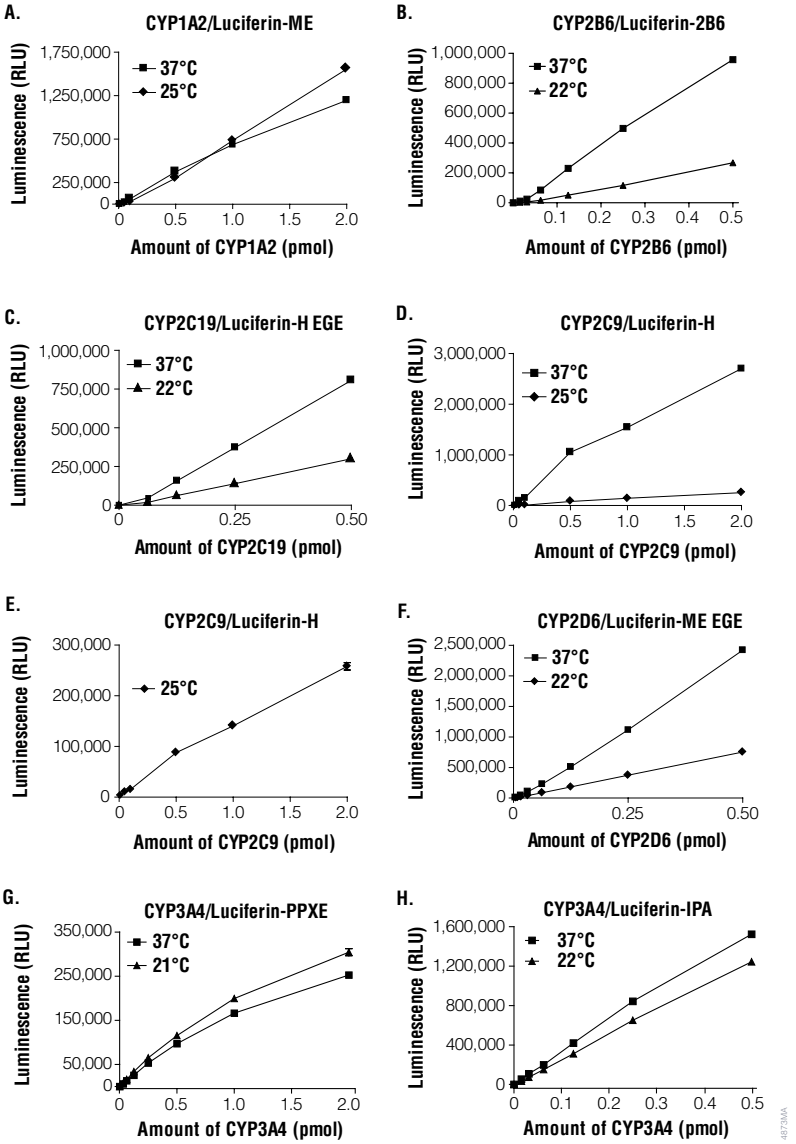

Figure 3. Titration of cytochrome P450 enzyme. P450-Glo™ Assays were performed with a range of CYP concentrations at 37°C and room temperature (20–25°C). Substrate concentrations and incubation times were as recommended in Table 2. Panel E shows in more detail data from the CYP2C9/Luciferin-H reaction performed at 25°C. Luminescence was measured using a GloMax® 96 Microplate Luminometer.

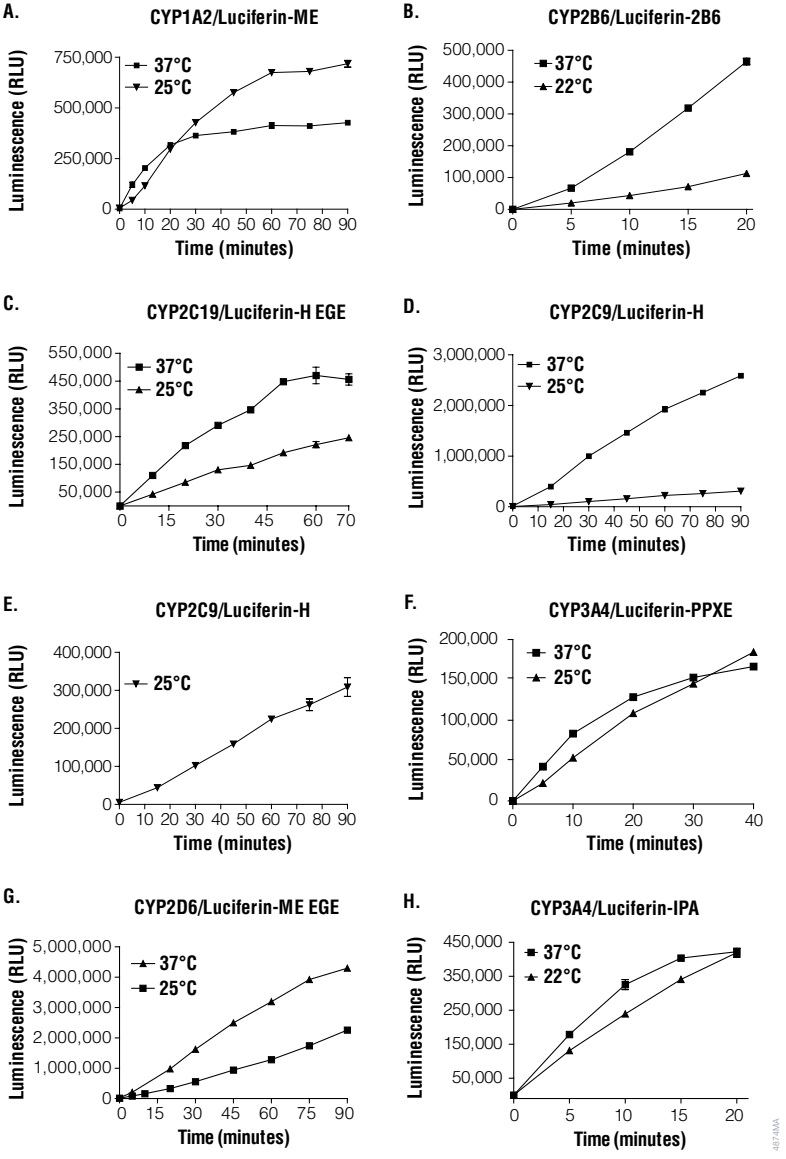

Figure 4. Incubation time and temperature. P450-Glo™ reactions (50µl) were performed with the enzyme and substrate concentrations indicated in Table 2. The P450-Glo™ Assays were incubated at room temperature (20–25°C) or 37°C for up to 90 minutes prior to adding the reconstituted Luciferin Detection Reagent. Panel E shows in more detail data from the CYP2C9/Luciferin-H reaction performed at 25°C. Luminescence was measured using a GloMax® 96 Microplate Luminometer (Panels A, B, D, E, F, G and H) or a POLARstar luminometer (BMG Labtech, Panel C).

4. General Overview of Protocol

Step 1. In a white opaque 96-well plate, combine one-fourth the final reaction volume of a 4X cytochrome P450 reaction mixture (e.g., 12.5µl in a 96-well plate) and an equal volume of the test compound or known inhibitor at a 4X concentration to give one-half of the final reaction volume. Perform a 10-minute pre-incubation. Add one-half the final volume of the 2X NADPH regeneration system (e.g., 25µl in a 96-well plate) to initiate the CYP reactions, and bring all components to their 1X target concentrations. Incubate at 37°C or room temperature for 10–45 minutes. See Table 2 for the recommended incubation times.

Step 2. Add the reconstituted Luciferin Detection Reagent. This stops the CYP reaction and initiates luminescence. Allow the signal to stabilize for 20 minutes at room temperature. Measure luminescence.

Materials to Be Supplied by the User

- 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, clear plates or black plates.
- luminometer or charge-coupled device (CCD) capable of reading multiwell plates
  (If using a multifunctional instrument, be sure to operate it in luminescence, **not** fluorescence, mode.)
- **optional:** multichannel pipette or automated pipetting station
- known CYP inhibitors as positive controls for inhibition (e.g., α-naphthoflavone, clopidogrel, sulfaphenazole, ketoconazole, quinidine or troglitazone, available from Sigma Aldrich)
- multiwell plate shaker for mixing plates (optional)
- D-luciferin (Beetle Luciferin, Potassium Salt, Cat.# E1601) (optional, for standard curve generation, Section 13)
- acetonitrile

The plate layout shown in Figure 5 can be used when studying the effects of test compounds or known CYP inhibitors on CYP activity. A single concentration of multiple compounds may be tested, or a range of concentrations for each compound may be tested to derive IC_{50} values. The plate layout shown in Figure 5 includes the appropriate controls.

- **Minus-P450 Control:** Contains the luminogenic P450-Glo™ substrate, Control Membranes, which lack CYP, and Potassium Phosphate Buffer. The values from these wells represent the CYP-independent background luminescence of the assay. The average of these values is subtracted from the luminescence of the CYP reactions to give the net CYP-dependent luminescence.
- **Control Inhibitor:** Contains one of the human CYP membrane preparations, luminogenic P450-Glo™ substrate, Potassium Phosphate Buffer and a known inhibitor. This control determines the capacity of the system to detect inhibition by test compounds. The recommended control inhibitors are 1µM α-naphthoflavone for CYP1A2, 1µM clopidogrel for CYP2B6, 2µM sulfaphenazole for CYP2C9, 10µM troglitazone for CYP2C19, 1µM quinidine for CYP2D6 and 1µM ketoconazole for CYP3A4.
- **Untreated:** Contains one of the human CYP membrane preparations, Potassium Phosphate Buffer and luminogenic P450-Glo™ substrate without known inhibitor or test compound. Values from these wells represent total CYP activity.
- **TC 1–TC 29:** Contains one of the human CYP membrane preparations, luminogenic P450-Glo™ substrate, Potassium Phosphate Buffer and test compound (TC). Luminescent values from these wells are compared to values from untreated control wells to ascertain the effect of the test compounds on CYP activity. A typical first-pass screening concentration of test compounds is 10µM.

	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

4857MA

TC=test compound

Figure 5. Plate layout for the P450-Glo™ Assays.

5. Preparing the Luciferin Detection Reagent

1. Equilibrate the Luciferin Detection Reagent (lyophilized) and Reconstitution Buffer or Reconstitution Buffer with esterase to room temperature. For CYP2B6, equilibrate the 2M D-Cysteine Solution to room temperature. Mix or vortex the D-cysteine solution as necessary to dissolve any content crystallized during –20°C storage. Use the Reconstitution Buffer with esterase to prepare the Luciferin Detection Reagent for CYP2C19, 2D6 and 3A4/Luciferin-IPA assays. Use the Reconstitution Buffer to prepare the Luciferin Detection Reagent for the other assays.

These two buffers are not interchangeable. To ensure use of the correct buffer for a given CYP assay, write the CYP of interest in the blank space provided on the Luciferin Detection Reagent label.

2. Transfer the entire contents of the 50ml bottle of Reconstitution Buffer or Reconstitution Buffer with esterase to the amber bottle containing the lyophilized Luciferin Detection Reagent. Mix by swirling or inverting several times to obtain a homogeneous solution. For the CYP2B6 assay, use the Reconstitution Buffer to reconstitute the lyophilized Luciferin Detection Reagent. Dilute the supplied 2M D-Cysteine Solution, a 500X stock solution, to a final concentration of 1X in reconstituted Luciferin Detection Reagent. To avoid foaming, do not vortex. 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. Store at –20°C for up to 3 months. Be sure to mix the thawed Luciferin Detection Reagent well before use.

6. CYP1A2 Assay Protocol

6.A. Preparing the Assay Components

1. Prepare the Luciferin Detection Reagent with Reconstitution Buffer as described in Section 5.
2. Thaw the CYP1A2 membranes and Control Membranes rapidly at 37°C. Upon thawing, immediately place them on ice. Mix well before use. Dispense unused membranes into single-use aliquots, and store at –70°C.
3. Thaw Luciferin-ME, Solutions A and B of the NADPH Regeneration System, Potassium Phosphate Buffer, 1M, and Luciferin-Free Water and store on ice. Protect substrate from light. Store unused substrate and Solutions A and B at –20°C.

   Note: A precipitate may form in Luciferin-ME upon freezing and thawing. Dissolve the precipitate by warming the vial to 37°C and vortex mixing.
4. For 96-well plates, consider the concentrations listed in Table 2 and prepare 12.5µl of 4X CYP1A2 reaction mixture for each well, as indicated in Table 3. For smaller well formats, scale reagent volumes as necessary. Use Table 3 to calculate the volume of each component needed for the 4X CYP1A2 reaction mixture. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells.

   Add components in the order shown in Table 3. Mix after each component is added. Store the 4X CYP1A2 reaction mixture on ice until ready to use.

Table 3. Preparation of the 4X CYP1A2 Reaction Mixture.

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
Luciferin-Free Water	6.0µl				
Potassium Phosphate Buffer, 1M	5.0µl				
5mM Luciferin-ME	1.0µl				
CYP1A2 membranes	0.5µl				
Final volume	**12.5µl**				

5. Prepare a separate 4X control reaction mixture for the minus-P450 control reactions by substituting Control Membranes for the CYP1A2 membranes. Adjust the protein concentration of reactions with Control Membranes to match that of the CYP1A2 reactions. See the component labels for lot-specific information on protein content. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells.
6. Prepare 25µl of 2X NADPH regeneration system for each well of a 96-well plate, as indicated in Table 4. For smaller well formats, scale reagent volumes as necessary. Use Table 4 to calculate the volume of each component by multiplying the volume per reaction by the number of reactions to be performed.

   To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells. Store the 2X NADPH regeneration system at room temperature until ready to use.

   Note: See Section 14 for a detailed description of the NADPH regeneration system.

Table 4. Preparation of the 2X NADPH Regeneration System.

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

7. Prepare test compounds and known inhibitor at a 4X concentration. To use α-naphthoflavone as a control CYP1A2 inhibitor, prepare a 1mM stock solution in acetonitrile or DMSO. Dilute this stock solution with water to 4µM.

If test compounds are diluted from concentrated stock solutions prepared in a solvent or buffer other than water (e.g., DMSO), include an equivalent amount of solvent in all control reactions to account for potential vehicle effects.

Note: The vehicle is defined as the solvent used to prepare the test compound stock solution plus any water used to further dilute the stock solutions to the 4X concentration.

6.B. Performing the CYP1A2 P450-Glo™ Assay

This protocol is written for a 96-well plate format. If you are using smaller well formats, scale reagent volumes accordingly.

1. Add up to 12.5µl of 4X test compound to the TC wells (Figure 5). If the volume of test compound is less than 12.5µl, add Luciferin-Free Water to bring the volume of each well to 12.5µl.
2. Add 12.5µl of Luciferin-Free Water or vehicle to untreated and minus-P450 control wells.
3. Add 12.5µl of 4µM α-naphthoflavone or another known CYP1A2 inhibitor at the appropriate concentration to control inhibitor wells.
4. Add 12.5µl of the 4X control reaction mixture to the minus-P450 control wells and 12.5µl of the 4X CYP1A2 reaction mixture to all other wells.
5. Mix briefly on a plate shaker or by tapping the plate.
6. Pre-incubate the plate at the desired reaction temperature (room temperature or 37°C) for 10 minutes.

   Note: Reactions can be performed at room temperature or 37°C. See Section 3.B.
7. Start reactions by adding 25µl of 2X NADPH regeneration system to all wells. Mix briefly on a plate shaker or by tapping the plate.
8. Incubate the plate at 37°C or room temperature for 10 minutes or 30 minutes, respectively.
9. Add 50µl of reconstituted Luciferin Detection Reagent to all wells. Mix briefly on a plate shaker or by tapping the plate.
10. Incubate the plate at room temperature for 20 minutes to stabilize the luminescent signal.

6.B. Performing the CYP1A2 P450-Glo™ Assay (continued)

11. Record luminescence using a plate-reading luminometer or CCD camera. Values are displayed as relative light units (RLU).

    Note: Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Relative light units (RLU) are arbitrary units that vary between instrument manufacturers and models. Do not use a fluorometer. Do not use filters with the luminometer.

7. CYP2B6 Assay Protocol

7.A. Preparing the Assay Components

1. Prepare the Luciferin Detection Reagent with Reconstitution Buffer and further supplement with D-cysteine as described in Section 5.

   Note: Mix or vortex the 2M D-Cysteine Solution as necessary to dissolve any content crystallized during –20°C storage.

2. Thaw the CYP2B6 membranes and Control Membranes rapidly at 37°C. Upon thawing, immediately place them on ice. Mix well before use. Dispense unused membranes into single-use aliquots, and store at –70°C.

3. Thaw Luciferin-2B6, Solutions A and B of the NADPH Regeneration System, Potassium Phosphate Buffer, 1M, and Luciferin-Free Water; store Luciferin-2B6 at room temperature and the other components on ice. Protect substrate from light. Store unused substrate and Solutions A and B at –20°C.

4. For 96-well plates, consider the concentrations listed in Table 2 and prepare 12.5µl of 4X CYP2B6 reaction mixture for each well, as indicated in Table 5. For smaller well formats, scale reagent volumes as necessary. Use Table 5 to calculate the volume of each component needed for the 4X CYP2B6 reaction mixture. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 100 wells.

   Add components in the order shown in Table 5. Mix after each component is added. Store the 4X CYP2B6 reaction mixture on ice until ready to use.

Table 5. Preparation of the 4X CYP2B6 Reaction Mixture.

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
Luciferin-Free Water	7.35µl				
Potassium Phosphate Buffer, 1M	5.0µl				
Luciferin-2B6, 3mM	0.05µl[1]				
CYP2B6 membranes	0.1µl				
Final volume	**12.5µl**				

[1]To prepare 4X CYP2B6 reaction mixture for fewer than 100 reactions, dilute the Luciferin-2B6, 3mM, stock to 0.3mM in water. For each 12.5µl of the 4X reaction mixture, add 0.5µl of Luciferin-2B6 and 6.9µl of Luciferin-Free Water.

5. Prepare a separate 4X control reaction mixture for the minus-P450 control reactions by substituting Control Membranes for the CYP2B6 membranes. Adjust the protein concentration of reactions with Control Membranes to match that of the CYP2B6 reactions. See the component labels for lot-specific information on protein content. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells.
6. Prepare 25µl of 2X NADPH regeneration system for each well of a 96-well plate, as indicated in Table 6. For smaller well formats, scale reagent volumes as necessary. Use Table 6 to calculate the volume of each component by multiplying the volume per reaction by the number of reactions to be performed.

   To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells. Store the 2X NADPH regeneration system at room temperature until ready to use.

   Note: See Section 14 for a detailed description of the NADPH regeneration system.

Table 6. Preparation of the 2X NADPH Regeneration System.

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

7. Prepare test compounds and known inhibitor at a 4X concentration. To use clopidogrel as a control CYP2B6 inhibitor, prepare a 1mM stock solution in acetonitrile or DMSO. Dilute this stock solution with water to 4µM.

If test compounds are diluted from concentrated stock solutions prepared in a solvent or buffer other than water (e.g., DMSO), include an equivalent amount of solvent in all control reactions to account for potential vehicle effects.

Note: The vehicle is defined as the solvent used to prepare the test compound stock solution plus any water used to further dilute the stock solutions to the 4X concentration.

7.B. Performing the CYP2B6 P450-Glo™ Assay

This protocol is written for a 96-well plate format. If you are using smaller well formats, scale reagent volumes accordingly.

1. Add up to 12.5µl of 4X test compound to the TC wells (Figure 5). If the volume of test compound is less than 12.5µl, add Luciferin-Free Water to bring the volume of each well to 12.5µl.
2. Add 12.5µl of Luciferin-Free Water or vehicle to untreated and minus-P450 control wells.
3. Add 12.5µl of 4µM clopidogrel or another known CYP2B6 inhibitor at the appropriate concentration to control inhibitor wells.

7.B. Performing the CYP2B6 P450-Glo™ Assay (continued)

4. Add 12.5µl of the 4X control reaction mixture to the minus-P450 control wells and 12.5µl of the 4X CYP2B6 reaction mixture to all other wells.
5. Mix briefly on a plate shaker or by tapping the plate.
6. Pre-incubate the plate at the desired reaction temperature (room temperature or 37°C) for 10 minutes.

   Note: Reactions can be performed at room temperature or 37°C. See Section 3.B.
7. Start reactions by adding 25µl of 2X NADPH regeneration system to all wells. Mix briefly on a plate shaker or by tapping the plate.
8. Incubate the plate at 37°C or room temperature for 10 minutes.
9. Add 50µl of reconstituted Luciferin Detection Reagent supplemented with D-cysteine to all wells. Mix briefly on a plate shaker or by tapping the plate.
10. Incubate the plate at room temperature for 20 minutes to stabilize the luminescent signal.
11. Record luminescence using a plate-reading luminometer or CCD camera. Values are displayed as relative light units (RLU).

    Note: Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Relative light units (RLU) are arbitrary units that vary between instrument manufacturers and models. Do not use a fluorometer. Do not use filters with the luminometer.

8. CYP2C9 Assay Protocol

8.A. Preparing the Assay Components

1. Prepare the Luciferin Detection Reagent with Reconstitution Buffer as described in Section 5.
2. Thaw the CYP2C9 membranes and Control Membranes rapidly at 37°C. Upon thawing, immediately place them on ice. Mix well before use. Dispense unused membranes into single-use aliquots, and store at –70°C.
3. Thaw Luciferin-H, Solutions A and B of the NADPH Regeneration System, Potassium Phosphate Buffer, 1M, and Luciferin-Free Water and store on ice. Protect substrate from light. Store unused substrate and Solutions A and B at –20°C.
4. For 96-well plates, consider the concentrations listed in Table 2 and prepare 12.5µl of 4X CYP2C9 reaction mixture for each well, as indicated in Table 7. For smaller well formats, scale reagent volumes as necessary. Use Table 7 to calculate the volume of each component needed for the 4X CYP2C9 reaction mixture. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells.

   Add components in the order shown in Table 7. Mix after each component is added. Store the 4X CYP2C9 reaction mixture on ice until ready to use.

Table 7. Preparation of the 4X CYP2C9 Reaction Mixture.

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
Luciferin-Free Water	9.75µl				
Potassium Phosphate Buffer, 1M	1.25µl				
5mM Luciferin-H	1.0µl				
CYP2C9 membranes	0.5µl				
Final volume	**12.5µl**				

5. Prepare a separate 4X control reaction mixture for the minus-P450 control reactions by substituting Control Membranes for the CYP2C9 membranes. Adjust the protein concentration of reactions with the Control Membranes to match that of the CYP2C9 reactions. See the component labels for lot-specific information on protein content. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells.

6. Prepare 25µl of 2X NADPH regeneration system for each well of a 96-well plate, as indicated in Table 8. For smaller well formats, scale reagent volumes as necessary. Use Table 8 to calculate the volume of each component by multiplying the volume per reaction by the number of reactions to be performed.

   To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells. Store the 2X NADPH regeneration system at room temperature until ready to use.

   Note: See Section 14 for a detailed description of the NADPH regeneration system.

Table 8. Preparation of the 2X NADPH Regeneration System.

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

7. Prepare test compounds and known inhibitor at a 4X concentration. To use sulfaphenazole as a control CYP2C9 inhibitor, prepare a 10mM stock solution in acetonitrile or DMSO. Dilute this stock solution with water to 40µM.

If test compounds are diluted from concentrated stock solutions prepared in a solvent or buffer other than water (e.g., DMSO), include an equivalent amount of solvent in all control reactions to account for potential vehicle effects.

Note: The vehicle is defined as the solvent used to prepare the test compound stock solution plus any water used to further dilute the stock solutions to the 4X concentration.

8.B. Performing the CYP2C9 P450-Glo™ Assay

This protocol is written for a 96-well plate format. If you are using smaller well formats, scale reagent volumes accordingly.

1. Add up to 12.5µl of 4X test compound to the TC wells (Figure 5). If the volume of test compound is less than 12.5µl, add Luciferin-Free Water to bring the volume of each well to 12.5µl.
2. Add 12.5µl of Luciferin-Free Water or vehicle to untreated and minus-P450 control wells.
3. Add 12.5µl of 40µM sulfaphenazole or another known CYP2C9 inhibitor at the appropriate concentration to control inhibitor wells.
4. Add 12.5µl of the 4X control reaction mixture to the minus-P450 control wells and 12.5µl of the 4X CYP2C9 reaction mixture to all other wells.
5. Mix briefly on a plate shaker or by tapping the plate.
6. Pre-incubate the plate at the desired reaction temperature (room temperature or 37°C) for 10 minutes.

   Note: Reactions can be performed at room temperature or 37°C. See Section 3.B.
7. Start reactions by adding 25µl of 2X NADPH regeneration system to all wells. Mix briefly on a plate shaker or by tapping the plate.
8. Incubate the plate at the desired temperature (room temperature or 37°C) for 30 minutes.
9. Add 50µl of reconstituted Luciferin Detection Reagent to all wells. Mix briefly on a plate shaker or by tapping the plate.
10. Incubate the plate at room temperature for 20 minutes to stabilize the luminescent signal.
11. Record luminescence using a plate-reading luminometer or CCD camera. Values are displayed as relative light units (RLU).

    Note: The luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Relative light units (RLU) are arbitrary units that vary between instrument manufacturers and models. Do not use a fluorometer. Do not use filters with the luminometer.

9. CYP2C19 Assay Protocol

9.A. Preparing the Assay Components

1. Prepare the Luciferin Detection Reagent with Reconstitution Buffer with esterase as described in Section 5.
2. Thaw the CYP2C19 membranes and Control Membranes rapidly at 37°C. Upon thawing, immediately place them on ice. Mix well before use. Dispense unused membranes into single-use aliquots, and store at –70°C.
3. Prepare a 10mM Luciferin-H EGE solution in acetonitrile: Dissolve 123µg of Luciferin-H EGE in 40µl of acetonitrile; mix vigorously to ensure complete solubilization of the substrate. Protect substrate from light. Store unused portion at –20°C.
4. Thaw Solutions A and B of the NADPH Regeneration System, Potassium Phosphate Buffer, 1M, and Luciferin-Free Water and store on ice. Store unused Solution A and Solution B at –20°C.

5. For 96-well plates, consider the concentrations listed in Table 2 and prepare 12.5µl of 4X CYP2C19 reaction mixture for each well. For smaller well formats, scale reagent volumes as necessary. Use Table 9 to calculate the volume of each component needed for the 4X CYP2C19 reaction mixture. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 100 wells.

   Add components in the order shown in Table 9. Mix after each component is added. Store the 4X CYP2C19 reaction mixture on ice until ready to use.

Table 9. Preparation of the 4X CYP2C19 Reaction Mixture.

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
Luciferin-Free Water[1]	9.7µl				
Potassium Phosphate Buffer, 1M	2.5µl				
10mM Luciferin-H EGE[1]	0.05µl				
CYP2C19 membranes	0.25µl				
Final volume	**12.5µl**				

[1]To prepare 4X CYP2C19 reaction mixture for fewer than 100 reactions, dilute the 10mM Luciferin-H EGE stock to 1.0mM in water. For each 12.5µl of the 4X reaction mixture, add 0.5µl of 1.0mM Luciferin-H EGE and 9.25µl of Luciferin-Free Water.

6. Prepare a separate 4X control reaction mixture for the minus-P450 control reactions by substituting Control Membranes for CYP2C19 membranes. Adjust the protein concentration of reactions with the Control Membranes to match that of the CYP2C19 reactions. See the component labels for lot-specific information on protein content. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells.

7. Prepare 25µl of 2X NADPH regeneration system for each well of a 96-well plate, as indicated in Table 10. For smaller well formats, scale reagent volumes as necessary. Use Table 10 to calculate the volume of each component by multiplying the volume per reaction by the number of reactions to be performed.

   To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells. Store the 2X NADPH regeneration system at room temperature until ready to use.

   Note: See Section 14 for a detailed description of the NADPH regeneration system.

Table 10. Preparation of the 2X NADPH Regeneration System.

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

9.A. Preparing the Assay Components (continued)

8. Prepare test compounds and known inhibitor at a 4X concentration. To use troglitazone as a control CYP2C19 inhibitor, prepare a 10mM stock solution in DMSO or acetonitrile. Dilute this stock solution with water to 40µM.

If test compounds are diluted from concentrated stock solutions prepared in a solvent or buffer other than water (e.g., DMSO), include an equivalent amount of solvent in all control reactions to account for potential vehicle effects.

Note: The vehicle is defined as the solvent used to prepare the test compound stock solution plus any water used to further dilute the stock solutions to the 4X concentration.

9.B. Performing the CYP2C19 P450-Glo™ Assay

This protocol is written for a 96-well plate format. If you are using smaller well formats, scale reagent volumes accordingly.

1. Add up to 12.5µl of 4X test compound to the TC wells (Figure 5). If the volume of test compound is less than 12.5µl, add Luciferin-Free Water to bring the volume of each well to 12.5µl.
2. Add 12.5µl of Luciferin-Free Water or vehicle to untreated and minus-P450 control wells.
3. Add 12.5µl of 40µM troglitazone or another known CYP2C19 inhibitor at the appropriate concentration to control inhibitor wells.
4. Add 12.5µl of the 4X control reaction mixture to the minus-P450 control wells and 12.5µl of the 4X CYP2C19 reaction mixture to all other wells.
5. Mix briefly on a plate shaker or by tapping the plate.
6. Pre-incubate the plate at the desired reaction temperature (room temperature or 37°C) for 10 minutes.

   Note: Reactions can be performed at room temperature or 37°C. See Section 3.B.
7. Start reactions by adding 25µl of 2X NADPH regeneration system to all wells. Mix briefly on a plate shaker or by tapping the plate.
8. Incubate the plate at 37°C or room temperature for 20 minutes or 30 minutes, respectively.
9. Add 50µl of reconstituted Luciferin Detection Reagent to all wells. Mix briefly on a plate shaker or by tapping the plate.

Use the Luciferin Detection Reagent prepared using the Reconstitution Buffer with esterase when performing the CYP2C19 assay.

10. Incubate the plate at room temperature for 20 minutes to stabilize the luminescent signal.
11. Record luminescence using a plate-reading luminometer or CCD camera. Values are displayed as relative light units (RLU).

    Note: The luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Relative light units (RLU) are arbitrary units that vary between instrument manufacturers and models. Do not use a fluorometer. Do not use filters with the luminometer.

10. CYP2D6 Assay Protocol

10.A. Preparing the Assay Components

1. Prepare the Luciferin Detection Reagent with Reconstitution Buffer with esterase as described in Section 5.
2. Thaw the CYP2D6 membranes and Control Membranes rapidly at 37°C. Upon thawing, immediately place them on ice. Mix well before use. Dispense unused membranes into single-use aliquots, and store at –70°C.
3. Prepare a 10mM Luciferin-ME EGE solution in acetonitrile. Dissolve 900µg of Luciferin-ME EGE in 265µl of acetonitrile; mix vigorously to ensure complete solubilization of the substrate. Protect substrate from light. Store unused portion at –20°C.
4. Thaw Solutions A and B of the NADPH Regeneration System, Potassium Phosphate Buffer, 1M, and Luciferin-Free Water and store on ice. Store unused Solution A and Solution B at –20°C.
5. For 96-well plates, consider the concentrations listed in Table 2 and prepare 12.5µl of 4X CYP2D6 reaction mixture for each well as indicated in Table 11. For smaller well formats, scale reagent volumes as necessary. Use Table 11 to calculate the volume of each component needed for the 4X CYP2D6 reaction mixture. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 50 wells.

   Add components in the order shown in Table 11. Mix after each component is added. Store the 4X CYP2D6 reaction mixture on ice until ready to use.

Table 11. Preparation of the 4X CYP2D6 Reaction Mixture.

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
Luciferin-Free Water[1]	7.1µl				
Potassium Phosphate Buffer, 1M	5.0µl				
10mM Luciferin-ME EGE[1]	0.15µl				
CYP2D6 membranes	0.25µl				
Final volume	**12.5µl**				

[1]To prepare 4X CYP2D6 reaction mixture for fewer than 50 reactions, dilute the 10mM Luciferin-ME EGE stock to 1.0mM in water. For each 12.5µl of the 4X reaction mixture, add 1.5µl of 1.0mM Luciferin-ME EGE and 5.75µl of Luciferin-Free Water.

6. Prepare a separate 4X control reaction mixture for the minus-P450 control reactions by substituting Control Membranes for the CYP2D6 membranes. Adjust the protein concentration of reactions with the Control Membranes to match that of the CYP2D6 reactions. See the component labels for lot-specific information about protein content. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells.

10.A. Preparing the Assay Components (continued)

7. Prepare 25µl of 2X NADPH regeneration system for each well of a 96-well plate, as indicated in Table 12. For a smaller well formats, scale reagent volumes as necessary. Use Table 12 to calculate the volume of each component by multiplying the volume per reaction by the number of reactions to be performed.

   To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells. Store the 2X NADPH regeneration system at room temperature until ready to use.

   Note: See Section 14 for a detailed description of the NADPH regeneration system.

Table 12. Preparation of the 2X NADPH Regeneration System.

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

8. Prepare test compounds and known inhibitor at a 4X concentration. To use quinidine as a control CYP2D6 inhibitor, prepare a 4µM stock in water.

If test compounds are diluted from concentrated stock solutions prepared in a solvent or buffer other than water (e.g., DMSO), include an equivalent amount of solvent in the control reactions to account for potential vehicle effects.

Note: The vehicle is defined as the solvent used to prepare the test compound stock solution plus any water used to further dilute the stock solutions to the 4X concentration.

10.B. Performing the CYP2D6 P450-Glo™ Assay

This protocol is written for a 96-well plate format. If you are using smaller well formats, scale reagent volumes accordingly.

1. Add up to 12.5µl of 4X test compound to the TC wells (Figure 5). If the volume of test compound is less than 12.5µl, add Luciferin-Free Water to bring the volume of each well to 12.5µl.
2. Add 12.5µl of Luciferin-Free Water or vehicle to untreated and minus-P450 control wells.
3. Add 12.5µl of 4µM quinidine or another known CYP2D6 inhibitor at the appropriate concentration to control inhibitor wells.
4. Add 12.5µl of the 4X control reaction mixture to the minus-P450 control wells and 12.5µl of the 4X CYP2D6 reaction mixture to all other wells.
5. Mix briefly on a plate shaker or by tapping the plate.
6. Pre-incubate the plate at the desired reaction temperature (room temperature or 37°C) for 10 minutes.

   Note: Reactions can be performed at room temperature or 37°C. See Section 3.B.

7. Start reactions by adding 25µl of 2X NADPH regeneration system to all wells. Mix briefly on a plate shaker or by tapping the plate.
8. Incubate the plate at 37°C or room temperature for 30 minutes or 45 minutes, respectively.
9. Add 50µl of reconstituted Luciferin Detection Reagent to all wells. Mix briefly on a plate shaker or by tapping the plate.

Use the Luciferin Detection Reagent prepared using the Reconstitution Buffer with esterase when performing the CYP2D6 assay.

10. Incubate the plate at room temperature for 20 minutes to stabilize the luminescent signal.
11. Record luminescence using a plate-reading luminometer or CCD camera. Values are displayed as relative light units (RLU).

    Note: The luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Relative light units (RLU) are arbitrary units that vary between instrument manufacturers and models. Do not use a fluorometer. Do not use filters with the luminometer.

11. CYP3A4 Assay Protocol

CYP3A4 systems are available with two distinct CYP3A4 substrates: Luciferin-IPA or Luciferin-PPXE. See Section 1, Notes for Table 1, for recommendations on choosing a CYP3A4 substrate.

11.A. Preparing the Assay Components

1. Prepare the Luciferin Detection Reagent for Luciferin-PPXE reactions with Reconstitution Buffer as described in Section 5. Prepare the Luciferin Detection Reagent for Luciferin-IPA reactions with Reconstitution Buffer with esterase.

   Note: The Reconstitution Buffer with esterase is required to process the CYP3A4/Luciferin-IPA reaction product.
2. Thaw the CYP3A4 membranes and Control Membranes rapidly at 37°C. Upon thawing, immediately place them on ice. Mix well before use. Dispense unused membranes into single-use aliquots, and store at –70°C.
3. Thaw the Luciferin-IPA or Luciferin-PPXE; Solutions A and B of the NADPH Regeneration System; Potassium Phosphate Buffer, 1M, and Luciferin-Free Water. For CYP3A4/Luciferin-PPXE reactions, thaw the 2M Tris-HCl (pH 7.5), 20X. Store Luciferin-PPXE and Luciferin-IPA at room temperature, protected from light. Store remaining components on ice. Store unused Luciferin-IPA and Solutions A and B at –20°C. Store unused Luciferin-PPXE at –70°C.
4. For CYP3A4/Luciferin-PPXE reactions, dilute the 2M Tris-HCl (pH 7.5), 20X, to 100mM with Luciferin-Free Water.

11.A. Preparing the Assay Components (continued)

5. For 96-well plates, consider the concentrations listed in Table 2 and prepare 12.5µl of 4X CYP3A4 reaction mixture for each well, as indicated in Table 13 or 14. Use these tables to calculate the volume of each component needed. For smaller well formats, scale reagent volumes as needed. Prepare enough of the 4X CYP3A4 Reaction Mixture With Luciferin-IPA or Luciferin-PPXE for at least 100 wells.

   Add components in the order shown in Table 13 or 14. Mix after each component is added. Store the 4X CYP3A4 reaction mixture on ice until ready to use.

For Luciferin-IPA prepare the 4X CYP3A4 reaction mixture with KPO_4 buffer and Luciferin-Free Water.

For Luciferin-PPXE, prepare the 4X CYP3A4 reaction mixture with 100mM Tris-HCl buffer (pH 7.5).

Table 13. Preparation of the 4X CYP3A4 Reaction Mixture With Luciferin-IPA.

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
Luciferin-Free Water	7.35µl				
Potassium Phosphate Buffer, 1M	5.0µl				
Luciferin-IPA, 3mM	0.05µl[1]				
CYP3A4 membranes	0.1µl				
Final volume	**12.5µl**				

[1]To prepare 4X CYP3A4 reaction mixture for fewer than 100 reactions, dilute the Luciferin-IPA, 3mM, stock to 0.3mM in water. For each 12.5µl of the 4X reaction mixture, and add 0.5µl of Luciferin-IPA and 6.9µl of Luciferin-Free Water.

Table 14. Preparation of the 4X CYP3A4 Reaction Mixture With Luciferin-PPXE.

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
100mM Tris-HCl (pH 7.5)	11.975µl				
50mM Luciferin-PPXE	0.025µl				
CYP3A4 membranes	0.5µl				
Final volume	**12.5µl**				

[1]To prepare 4X CYP3A4 reaction mixture for fewer than 100 reactions, dilute the 50mM Luciferin-PPXE stock to 5mM in water. For each 12.5µl of the 4X reaction mixture, and add 0.25µl of Luciferin-PPXE and 11.75µl of Luciferin-Free Water.

6. Prepare a separate 4X control reaction mixture for the minus-P450 control reactions by substituting Control Membranes for the CYP3A4 membranes.

   For Luciferin-IPA prepare the control mixture with KPO_4 buffer.

   For Luciferin-PPXE prepare the control mixture with 100mM Tris-HCl buffer (pH 7.5).

   Adjust the protein concentration of reactions with the Control Membranes to match that of the CYP3A4 reactions. See the component labels for lot-specific information about protein content. To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells.

7. Prepare 25µl of the 2X NADPH regeneration system for each well of a 96-well plate without KPO_4 buffer for Luciferin-IPA (Table 15) or with KPO_4 buffer for Luciferin-PPXE reactions (Table 16). For smaller well formats, scale reagent volume as necessary. Use Tables 15 and 16 to calculate the volume of each component by multiplying the volume per reaction by the number of reactions to be performed.

   To avoid errors due to pipetting small volumes, prepare enough of the mixture for at least 10 wells. Store the 2X NADPH regeneration system at room temperature until ready to use.

   Note: See Section 14 for a detailed description of the NADPH regeneration system.

Table 15. Preparation of the 2X NADPH Regeneration System for Luciferin-IPA Reactions.

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

Table 16. Preparation of the 2X NADPH Regeneration System for Luciferin-PPXE Reactions.

Component	Volume Per Reaction	×	Number of Reactions	=	Total Volume
Luciferin-Free Water	12.0µl				
Potassium Phosphate Buffer, 1M	10.0µl				
Solution A	2.5µl				
Solution B	0.5µl				
Final volume	**25.0µl**				

11.A. Preparing the Assay Components (continued)

8. Prepare test compounds and known inhibitor at a 4X concentration. To use ketoconazole as a control CYP3A4 inhibitor, prepare a 5mM stock solution in acetonitrile. Dilute this stock solution with water to 20µM.

If test compounds are diluted from concentrated stock solutions prepared in a solvent or buffer other than water (e.g., DMSO), include an equivalent amount of solvent in all control reactions to account for potential vehicle effects. DMSO is a known inhibitor of CYP3A4 reactions (7). Relative sensitivities of the CYP3A4 reactions are Luciferin-IPA>Luciferin-PPXE. Acetonitrile, methanol and ethanol do not substantially affect the assays at concentrations below about 2%.

Note: The vehicle is defined as the solvent used to prepare the test compound stock solution plus any water used to further dilute the stock solutions to the 4X concentration.

11.B. Performing the CYP3A4 P450-Glo™ Assay

This protocol is written for a 96-well plate format. For smaller well formats, scale reagent volumes as necessary.

1. Add up to 12.5µl of 4X test compound to the TC wells (Figure 5). If the volume of test compound is less than 12.5µl, add Luciferin-Free Water to bring the volume of each well to 12.5µl.
2. Add 12.5µl of Luciferin-Free Water or vehicle to untreated and minus-P450 control wells.
3. Add 12.5µl of 20µM ketoconazole or another known CYP3A4 inhibitor to control inhibitor wells.
4. Add 12.5µl of the 4X control reaction mixture to the minus-P450 control wells and 12.5µl of the 4X CYP3A4 reaction mixture to all other wells.
5. Mix briefly on a plate shaker or by tapping the plate.
6. Pre-incubate the plate at the desired reaction temperature (room temperature or 37°C) for 10 minutes.

   Note: Reactions can be performed at room temperature or 37°C. See Section 3.B.
7. Start reactions by adding 25µl of 2X NADPH regeneration system to all wells. Mix briefly on a plate shaker or by tapping the plate.
8. Incubate CYP3A4/Luciferin-IPA assays at room temperature or 37°C for 10 minutes.

   Incubate CYP3A4/Luciferin-PPXE assays at 37°C or room temperature for 15 minutes or 30 minutes, respectively.
9. Add 50µl of reconstituted Luciferin Detection Reagent to all wells. Mix briefly on a plate shaker or by tapping the plate.

Use the Luciferin Detection Reagent prepared using the Reconstitution Buffer when performing 3A4/Luciferin-PPXE assays. Use the Luciferin Detection Reagent prepared using the Reconstitution Buffer with esterase when performing 3A4/Luciferin-IPA assays.

10. Incubate the plate at room temperature for 20 minutes to stabilize the luminescent signal.

11. Record luminescence using a plate-reading luminometer or CCD camera. Values are displayed as relative light units (RLU).

    Note: Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Relative light units (RLU) are arbitrary units that vary between instrument manufacturers and models. Do not use a fluorometer. Do not use filters with the luminometer.

12. Results

Calculate net CYP-dependent luminescence by subtracting the average luminescence of the minus-P450 control reactions from the CYP-containing reactions. The net signals from untreated CYP reactions represent total CYP activity. Changes from the average net signal of untreated CYP reactions for reactions with a known inhibitor or test compound reflect the modulation of CYP activity by these compounds. Changes will typically be seen as decreases due to CYP inhibition. However, some compounds may cause signals to increase because they exhibit positive cooperativity with the CYP substrate. This phenomenon has been reported for CYP3A4 and CY2C9 (8,9).

The magnitude of luminescent signal is proportional to D-luciferin concentration. Data may be expressed directly in relative light units, or the values may be converted to reaction rates by dividing luminescence (in RLU) by reaction time and CYP amount. For example, obtaining 100,000RLU from a 20-minute reaction with 0.5pmol of CYP corresponds to a specific activity of 10,000RLU/pmol CYP/minute. Alternatively, RLU can be converted to a corresponding D-luciferin concentration in assays that include a D-luciferin standard curve (Figure 6). However, to detect CYP inhibition at a single concentration of a test compound or measure the IC_{50} or K_i value for an inhibitor, it is not necessary to convert RLU to D-luciferin concentration.

Note: IC_{50} refers to the concentration of a compound that reduces CYP activity to the midpoint of the full inhibition curve. In the case of competitive inhibition, $IC_{50} = 2K_i$ when the substrate is present at the K_m concentration, as per the relationship: $K_i = IC_{50}/ [1 + (\text{substrate concentration}/K_m)]$.

If IC_{50} values obtained for competitive inhibitors with the P450-Glo™ Assays will be compared to IC_{50} values obtained with other CYP assays, you must consider this relationship and note that direct IC_{50} comparisons should be made only when the respective substrates are present at their K_m concentrations. Also note that, because inhibition of CYP3A4 and 2C9 is substrate-dependent, it is unrealistic to expect matching K_i values for a given inhibitor against every substrate of these enzymes (8–11).

12. Results (continued)

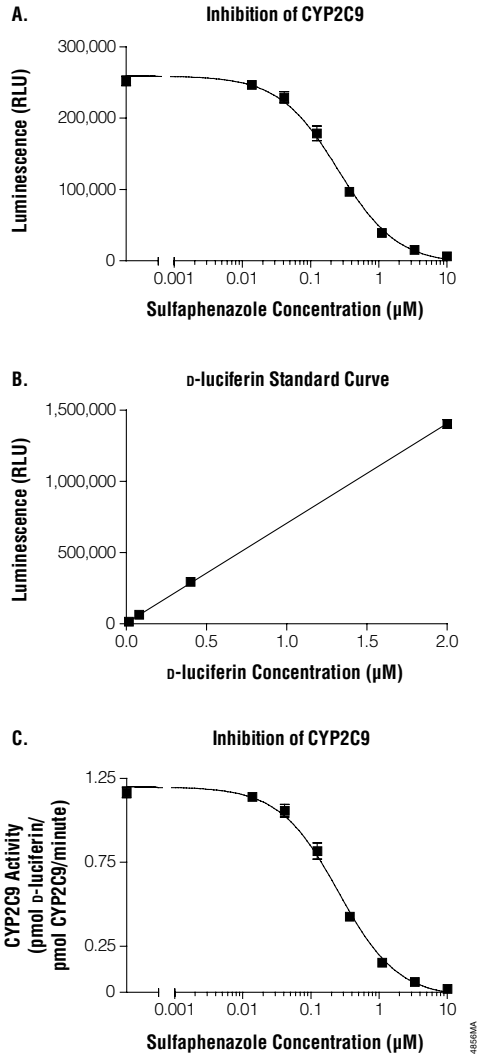

Figure 6. Representative P450-Glo™ Assay data. CYP2C9 reactions were performed in the presence or absence of the CYP2C9 inhibitor sulfaphenazole as described in Section 8. **Panel A.** The inhibition of CYP2C9 by sulfaphenazole is expressed in terms of RLU. **Panel B.** A D-luciferin standard curve was performed in parallel with CYP2C9 reactions, as described in Section 13, and analyzed by linear regression. $r^2 = 0.999$. **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 were then used to calculate CYP2C9 reaction rates (pmol D-luciferin/pmol CYP2C9/minute). The IC_{50} value derived from Panels A and C for inhibition of CYP2C9 by sulfaphenazole is 0.2µM. Luminescence was measured using a POLARstar luminometer (BMG Labtech).

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

The concentration of D-luciferin generated by CYP in P450-Glo™ Assays can be determined by comparing the luminescence from samples to 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 6. Standard curve measurements should be performed at the same time as samples. Use the plate layout shown in Figure 7. By comparing signals from CYP reactions to those from the D-luciferin standards, the quantity of D-luciferin generated by CYP can be determined.

See Section 13.B for an important consideration regarding quantifying signals from Luciferin-PPXE reactions.

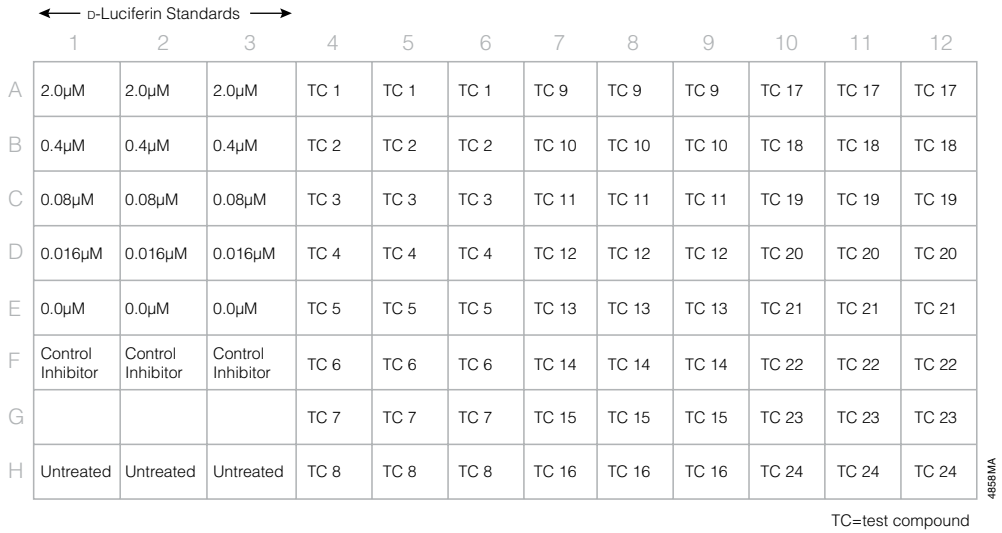

Figure 7. Plate layout for assays with a D-luciferin standard curve.

13.A. Generating a D-Luciferin Standard Curve

Prepare the D-luciferin stock solutions and D-luciferin standards at a location separate from where the P450-Glo™ Assays are performed. **Because of the high 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.
   ii. Pipette 900µl of water into the 8µM tube and 800µl of water into the other three tubes.

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

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.

4. Prepare the 4X CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 or CYP3A4 reaction mixtures, 4X control reaction mixture and 2X NADPH regeneration system as described in Section 6, 7, 8, 9, 10 or 11. 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 sample labeled as 0µM is equivalent to the minus-P450 control in Figure 5.

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 Sections 6, 7, 8, 9, 10 and 11 for CYP1A2, 2B6, 2C9, 2C19, 2D6 and 3A4 assays, respectively.

13.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 luminescence (in RLU).
- Interpolate CYP-generated D-luciferin concentrations in test samples by comparing their RLU values to 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 should 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 arrive at the true values, multiply the interpolated values by two.

14. 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 as a 20X concentrate. 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 electron source 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 NADPH 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 systems for use with CYP1A2, 2B6, 2C9, 2C19, 2D6 and 3A4 with Luciferin-IPA contain 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 (prepared by the user) for use with CYP3A4 with 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 NADPH regeneration system in P450-Glo™ Assays. The final concentration of NADPH in the CYP assay should be 100µM. NADPH can be purchased from Sigma-Aldrich and other chemical suppliers.

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.

15. K_m Measurements

The K_m value for a given CYP may vary somewhat between enzyme preparations (6). The concentrations of P450-Glo™ substrates recommended here are representative K_m concentrations for recombinant CYP enzyme preparations. When measuring K_m, Luciferin-H, Luciferin-ME, Luciferin-H EGE and Luciferin-ME EGE caused a partial inhibition of luciferase at the upper end of the concentration ranges tested, and thus diminished the brightness of the detection step. Such luciferase inhibition is not observed with Luciferin-2B6, Luciferin-IPA or Luciferin-PPXE. To measure a K_m value for a luminogenic P450-Glo™ Assay, we compensated for the inhibition of luciferase by the former P450-Glo™ substrates. Without compensation, luciferase is less sensitive when detecting luciferin at the higher substrate concentrations, resulting in underestimates of K_m and V_{max} values. For the K_m values reported in Table 2, compensation for luciferase inhibition in CYP1A2, 2C9, 2C19 and 2D6 reactions was made by performing CYP reactions at a range of substrate concentrations, adding the reconstituted Luciferin Detection Reagent to stop the reactions, 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 equal across the range of substrate concentrations. No compensation was made for substrate loss 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).

16. 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 of 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 reporter gene assays. • Decontaminate work surfaces by wiping with clean water. Rinse pipettes and other labware with distilled water multiple times. For automated dispensing systems, replace 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.
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.

Symptoms	Causes and Comments
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 and/or substrates to a surface that has been treated for enhanced hydrophobicity. Reactions with Luciferin-IPA are sensitive to this effect.
	The wrong buffer was used to resuspend the Luciferin Detection Reagent (lyophilized). Use the reagent labeled as Reconstitution Buffer for CYP1A2, 2B6, 2C9 and 3A4/Luciferin-PPXE assays; use the reagent labeled as Reconstitution Buffer with esterase for CYP3A4/Luciferin-IPA, 2C19 and 2D6 assays. These two buffers are not interchangeable. Label the blank space provided on the Luciferin Detection Reagent label with the assay name to ensure the correct buffer is used.
	Low CYP activity in enzyme preparation. • Store the CYP membranes at −70°C. Dispense the membranes into single-use aliquots to avoid multiple freeze-thaw cycles. • Thaw the CYP membranes immediately before use. Extended incubations on ice or at room temperature may lead to enzyme inactivation. • Mix the CYP membranes well before use. • For CYP2C9, incubate the assays at 37°C rather than at room temperature. Stronger signals are always generated at 37°C.
Low luminescent signal from CYP2B6 assay	The Luciferin Detection Reagent was not supplemented with D-Cysteine. D-Cysteine is required to convert the product of CYP2B6/Luciferin-2B6 reaction into D-luciferin.
Unexpected inhibition of CYP enzyme	Inhibition of CYP by test compound vehicle. Compare the luminescence from reactions with and without vehicle. A decrease in luminescence in the presence of the vehicle indicates CYP inhibition. Minimize solvent concentration, or use a different solvent to dissolve test compounds.
	Inhibition of CYP by test compound vehicle. DMSO is a known CYP3A4 inhibitor (6). Reactions with Luciferin-IPA and Luciferin-PPXE have minimal sensitivity to inhibition by DMSO.

16. Troubleshooting (continued)

Symptoms	Causes and Comments
Unexpected inhibition of P450-Glo™ Assay	P450-Glo™ substrate formed a precipitate upon thawing or dilution in aqueous mixtures. • Briefly warm thawed substrate to 37°C, then vortex to dissolve the substrate. • Potassium Phosphate Buffer, 1M, was added directly to the 4X CYP3A4/Luciferin-PPXE reaction mixtures. The 4X potassium phosphate concentration for CYP3A4 reactions is 800mM, and this may cause the substrate to precipitate. By introducing the Potassium Phosphate Buffer, 1M, to the reaction as part of the NADPH regeneration mixture, the substrate is not exposed to the high potassium phosphate concentration, and precipitation is avoided.
	Luciferase or esterase inhibition. Screen compounds using multiple CYP enzymes. Inhibition of only a subset of the enzymes indicates that the test compound is not a luciferase or esterase inhibitor.
	Luciferase or esterase inhibition. Luciferase is used to generate luminescence in P450-Glo™ Assays. A mixture of porcine esterases is used in the CYP3A4/Luciferin-IPA, CYP2C19 and CYP2D6 Luciferin Detection Reagent to process the products of the respective reactions. The potential for inhibition of luciferase or esterase is minimized by maintaining high enzyme concentrations and using reaction chemistries that reduce the effects of potential inhibitors. For example, 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 samples, 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 at room temperature for 10 minutes, 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 (continued)	If luciferase inhibition has been ruled out as a possible cause, perform the following test for esterase inhibition (CYP2C19, 2D6 and CYP3A4/Luciferin-IPA assays only). Perform CYP2C19, 2D6 or CYP3A4 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.
Luciferin-PPXE substrate forms precipitate in the 4X CYP3A4 Reaction Mixture With Luciferin-PPXE	Use 100mM Tris-HCl (pH 7.5), not water, to prepare the 4X CYP3A4 Reaction Mixture With Luciferin-PPXE. Luciferin-PPXE has greater solubility in Tris-HCl.
	When preparing the 4X CYP3A4 Reaction Mixture With Luciferin-PPXE, mix the Luciferin-PPXE and Tris-HCl (pH 7.5) immediately upon combining.

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17. Appendix

17.A. References

1. Cali, J.J. *et al.* (2006) Luminogenic cytochrome P450 assays. *Expert Opin. Drug Metab. Toxicol.* **2**, 629–45.
2. Cali, J.J. *et al.* (2012) Bioluminescent assays for ADME evaluation: dialing in CYP selectivity with luminogenic substrates. *Expert Opin Drug Metab Toxicol.* **8**, 1115–30.
3. Meisenheimer P.L. *et al.* (2011) Proluciferin acetals as bioluminogenic substrates for cytochrome P450 activity and probes for CYP3A inhibition. *Drug Metab Dispos.* **39**, 2403–10.
4. Lee, C.R., Goldstein, J.A. and Pieper, J.A. (2002) Cytochrome P450 2C9 polymorphisms: A comprehensive review of the in-vitro and human data. *Pharmacogenetics* **12**, 251–63.
5. Crespi, C.L. *et al.* (1995) Comparison of substrate metabolism by wildtype CYP2D6 protein and a variant containing methionine, not valine, at position 374. *Pharmacogenetics* **5**, 234–43.
6. Wienkers, L.C. and Hutzler, J.M. (2002) Screening for cytochrome P450 inhibitors. *Curr. Drug Disc.* **June**, 23–6.
7. Busby, W.F., Jr., Ackermann, J.M. and Crespi, C.L. (1999) Effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on in vitro activities of cDNA-expressed human cytochrome P-450. *Drug Metab. Dispos.* **27**, 246–9.
8. Ueng, Y.F. *et al.* (1997) Cooperativity in oxidations catalyzed by cytochrome P450 3A4. *Biochemistry* **36**, 370–81.
9. Hutzler, J.M., Hauer, M.J. and Tracy, T.S. (2001) Dapsone activation of CYP2C9-mediated metabolism: Evidence for activation of multiple substrates and a two-site model. *Drug Metab. Dispos.* **29**, 1029–34.
10. Korzekwa, K.R. *et al.* (1998) Evaluation of atypical cytochrome P450 kinetics with two-substrate models: Evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry* **37**, 4137–47.
11. Stresser, D.M. *et al.* (2000) Substrate-dependent modulation of CYP3A4 catalytic activity: Analysis of 27 test compounds with four fluorometric substrates. *Drug Metab. Dispos.* **28**, 1440–8.

17.B. Composition of Buffers and Solutions

Luciferin-2B6, 3mM

3mM Lucferin-2B6 in DMSO

Luciferin-H, 5mM

5mM Luciferin-H in 5mM sodium citrate buffer (pH 5.5)

Luciferin-H EGE

Luciferin-H EGE is supplied lyophilized. Once resuspended, Luciferin-H EGE is 10mM in acetonitrile.

Luciferin-IPA, 3mM

3mM Luciferin-IPA in DMSO

Luciferin-ME, 5mM

5mM Luciferin-ME in 5mM sodium citrate buffer (pH 5.5)

Luciferin-ME EGE

Luciferin-ME EGE is supplied lyophilized. Once resuspended, Luciferin-ME EGE is 10mM in acetonitrile.

Luciferin-PPXE, 50mM

50mM Luciferin-PPXE in DMSO plus 0.05N HCl

Luciferin-Free Water

A contaminant-free supply of water for making necessary dilutions.

Potassium Phosphate Buffer, 1M (pH 7.4)

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

Adjust the pH to 7.4 ± 0.1 at 25°C.

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)

Promega

17.C. Related Products

Product	Size	Cat.#
Beetle Luciferin, Potassium Salt	5mg	E1601
NADPH Regeneration System	1,000 assays	V9510

Additional sizes of Beetle Luciferin, Potassium Salt, available.

Cytochrome P450 Assays

Product	Size	Cat.#
P450-Glo™ CYP1A1 Assay	10ml	V8751
	50ml	V8752
P450-Glo™ CYP1A2 Assay	10ml	V8771
	50ml	V8772
P450-Glo™ CYP1A2 Induction/Inhibition Assay	10ml	V8421
	50ml	V8422
P450-Glo™ CYP1B1 Assay	10ml	V8761
	50ml	V8762
P450-Glo™ CYP2B6 Assay	10ml	V8321
	50ml	V8322
P450-Glo™ CYP2C8 Assay	10ml	V8781
	50ml	V8782
P450-Glo™ CYP2C9 Assay	10ml	V8791
	50ml	V8792
P450-Glo™ CYP2C19 Assay	10ml	V8881
	50ml	V8882
P450-Glo™ CYP2D6 Assay	10ml	V8891
	50ml	V8892
P450-Glo™ CYP3A4 Assay	10ml	V8801
	50ml	V8802
P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO-Tolerant Assay	10ml	V8911
	50ml	V8912
P450-Glo™ CYP3A4 Assay (Luciferin-IPA)	10ml	V9001
	50ml	V9002

Luciferin Detection Reagents

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

Additional sizes available.

Monoamine Oxidase Assay

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

Oxidative Stress Assays

Product	Size	Cat.#
GSH-Glo™ Assay	10ml	V6911
GSH/GSSG-Glo(TM) Assay	10ml	V6611
ROS-Glo(TM) H2O2 Assay	10ml	G8820
Griess Reagent System	1,000 assays	G2930

Luminometers

Product	Size	Cat.#
GloMax® 20/20 Luminometer	1 each	E5311
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500
GloMax® Navigator System	1 each	GM2000

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay (ATP)	10ml	G7570
RealTime-Glo™ MT Cell Viability Assay	100 assays	G9711
CellTiter-Glo® 2.0 Cell Viability Assay	10ml	G9241
CellTiter-Glo® 3D Cell Viability Assay	10ml	G9681
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080

Available in additional sizes.

17.C. Related Products (continued)

Apoptosis Assays

Product	Size	Cat.#
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011
Caspase-Glo® 3/7 Assay	100ml	G8092
Caspase-Glo® 8 Assay	100ml	G8202
Caspase-Glo® 9 Assay	100ml	G8212

Available in additional sizes.

Cytotoxicity Assays Section

Product	Size	Cat.#
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CellTox™ Green Cytotoxicity Assay	10ml	G8741

Available in additional sizes.

18. Summary of Changes

The following change was made to the 3/22 version of this document:

1. Removed discontinued product Cat.# V9800, affecting Sections 1, 3, 11 and 17.B.
2. Revised Section 17.C.
3. Updated disclaimers.
4. Updated cover page.

[a]European Pat. No. 1131441 and Japanese Pat. No. 4520084.

[b]U.S. Pat. No. 8,551,721 and other patents pending.

[c]U.S. Pat. No. 8,592,172 and other patents pending.

[d]U.S. Pat. No. 8,288,559 and other patents pending.

[e]U.S. Pat. Nos. 7,692,022 and 8,106,052 and other patents pending.