P450-Glo™ CYP3A4 Assay with Luciferin-IPA for Biochemical Samples

Instructions for Use of Products V9001and V9002.

Quick Protocol

The P450-Glo™ CYP3A4 Assay with Luciferin-IPA substrate can be used to selectively measure recombinant CYP3A4 activity. Significant activity of other human CYPs is not detected with Luciferin-IPA substrate.

These instructions are for biochemical assays performed in 96-well plates with a total reaction volume of 50µl. For smaller plate formats, scale reagent volumes proportionately. For detailed instructions, including plate setup, see the *P450-Glo™ Assays Technical Bulletin* #TB325, available at: **www.promega.com/protocols/**

Table 1. P450-Glo™ CYP3A4 Biochemical Assay Components.

Component	Volume
Test compound	12.5µl
4X CYP reaction mixture	12.5µl
2X NADPH	25µl
Luciferin Detection Reagent	50µl

Materials to Be Supplied By the User:

- NADPH Regeneration System (Cat.# V9510)
- 1M KPO_4 buffer (pH 7.4)
- distilled or deionized water.
- active CYP preparation that includes CYP3A4 reductase (see the *P450-Glo™ Assays Technical Bulletin* #TB325 for supplier information)
- preparation that lacks CYP activity for the minus-P450 control reactions
- 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.

Reagent Preparation

Preparing the Reconstituted Luciferin Detection Reagent

1. Equilibrate the lyophilized Luciferin Detection Reagent and the Reconstitution Buffer with esterase to room temperature.
2. Transfer the contents of one bottle of Reconstitution Buffer with Esterase to one amber bottle containing the lyophilized Luciferin Detection Reagent. 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 (20°C to 25°C) for 24 hours or at +2°C to +10°C for 1 week without loss of activity. For long-term storage, store at –30°C to –10°C for up to 3 months. Be sure to mix the thawed Luciferin Detection Reagent well before use.

Reagent Preparation (continued)

Preparing the 2X NADPH Regeneration System

1. Prepare the 2X NADPH Regeneration System on the day of use, as directed in Table 2. Store at room temperature until ready to use.

Table 2. Preparing the 2X NADPH Regeneration System.

Component	Volume Per Reaction
Water	22.0µl
Solution A	2.5µl
Solution B	0.5µl
Final Volume	**25.0µl**

Preparing the 4X CYP Reaction Mixture

1. Thaw the Luciferin-IPA substrate and keep at room temperature until ready to use.

   Note: Unused Luciferin-IPA substrate should be protected from light and stored at –30°C to –10°C.

2. Thaw the CYP preparation rapidly at 37°C, then place on ice.

   Note: Thaw CYP Preparations immediately before use to minimize enzyme instability. We suggest dispensing the membranes into smaller volume aliquots and storing at –70°C to minimize freeze-thaw cycles.

3. Prepare 12.5µl of 4X CYP reaction mixture for each CYP reaction using the concentration of each component listed in Table 3.

   Note: Use water to bring the reaction mixture to the final volume. 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.

Table 3. Preparing the 4X Reaction Mixtures.

Amount of CYP/12.5µl[1]	KPO_4 Concentration	Substrate Concentration
0.1pmol	400mM	12µM Luciferin-IPA

[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.

4. Similarly, prepare 12.5µl of 4X Control Reaction Mixture for each minus-P450 control reaction using the concentration of each component listed in Table 3. For this reaction mixture, use an equivalent amount of membrane protein from a membrane preparation that lacks CYP activity. Store mixture on ice until ready to use.

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P450-Glo™ CYP3A4 Assay with Luciferin-IPA Protocol

Note: See section 4.E in the *P450-Glo™ Assays Technical Bulletin* #TB325 for detailed information on suggested controls and plate layout.

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 nontreated and minus-P450 control wells.

   Note: Keep organic solvent to a minimum to avoid potential effects on CYP activities. Importantly, DMSO is a known inhibitor of many CYP3A4 reactions. CYP3A4 reactions with Luciferin-IPA show little or no sensitivity to DMSO at or below 0.25%, nor to acetonitrile, methanol or ethanol at or below 1.0%.

2. **CYP reactions:** Add 12.5µl of the 4X CYP reaction mixture to each well. Mix gently.
   Minus-P450 control reactions: Add 12.5µl of the 4X control reaction mixture to each well. Mix gently.
3. Incubate the plate at 37°C or room temperature for 10 minutes.
4. Start CYP reactions by adding 25µl of 2X NADPH regeneration system to each reaction. Mix briefly.
5. Incubate the plate at the same temperature used during the pre-incubation step (Step 3) for 10 minutes.
6. Add 50µl of reconstituted Luciferin Detection Reagent to each reaction.
7. Mix the plate for 10 seconds on an orbital shaker or by gently tapping the plate.
8. Incubate the plate at room temperature (20°C to 25°C) for 20 minutes to stabilize the luminescent signal.
9. Record luminescence.

   Note: Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline.

10. Calculate net luminescence and analyze assay results.

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