# P450-Glo™ CYP3A4 Assay (Luciferin-IPA)

INSTRUCTIONS FOR USE OF PRODUCTS V9001 AND V9002.

Quick PROTOCOL

These protocols are written for CYP3A4/Luciferin-IPA assays in a 96-well plate format. For smaller well formats, scale reagent volumes as necessary. For more information about the P450-Glo™ CYP3A4 Assay (Luciferin-IPA), see the *P450-Glo™ Assays Technical Bulletin* #TB325, which is available at: **www.promega.com/tbs/**

## Preparing the CYP3A4/Luciferin-IPA Assay Reagents

1. Equilibrate the Reconstitution Buffer with esterase to room temperature.
2. Transfer the contents of one bottle of Reconstitution Buffer with esterase to the amber bottle containing the lyophilized Luciferin Detection Reagent. Label the blank space on the Luciferin Detection Reagent label with the appropriate CYP name to ensure the correct Luciferin Detection Reagent is used.
3. Mix by swirling or inverting several times to obtain a homogeneous solution. Do not vortex.
   **Note:** The reconstituted Luciferin Detection Reagent can be stored at room temperature for 24 hours or at 4°C for 1 week without loss of activity. For long-term storage, store at –20°C for up to 3 months.
4. Thaw the Luciferin-IPA substrate, and keep it at room temperature while setting up assays. Keep Luciferin-IPA protected from light. Luciferin-IPA is provided as a solution in DMSO.
5. Prepare 25µl of 2X NADPH regeneration system for each well by combining 22.0µl of Luciferin-Free Water, 2.5µl of Solution A and 0.5µl of Solution B for each reaction.
6. Prepare 12.5µl of 4X CYP3A4 reaction mixture for each well by combining 5.0µl of Potassium Phosphate Buffer, 1M, 0.05µl of 3mM Luciferin-IPA and enough CYP preparation to supply 0.1pmol of CYP3A4.

   For each minus-P450 control reaction, prepare 12.5µl of 4X control reaction mixture by combining 5.0µl of Potassium Phosphate Buffer, 1M, 0.05µl of 3mM Luciferin-IPA and an equivalent amount of protein from a membrane preparation that lacks CYP activity.

   Store the 4X CYP3A4 reaction mixture and 4X control reaction mixture on ice until ready to use.
7. Prepare test compounds and known inhibitor at a 4X concentration using luciferin-free water.

## Performing CYP3A4/Luciferin-IPA P450-Glo™ Biochemical Assays

The concentration of D-luciferin generated by CYP in P450-Glo™ Assays can be determined by comparing the luminescence from CYP reactions to that from a D-luciferin standard curve. Refer to the *P450-Glo™ Assays Technical Bulletin* #TB325 for more information about quantifying P450-Glo™ signals using D-luciferin standard curves.

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 Luciferin-Free Water to bring the volume of each well to 12.5µl. For controls without a test compound, add 12.5µl of Luciferin-Free Water or test compound vehicle to each well.
2. **CYP reactions:** Add 12.5µl of the 4X CYP3A4 reaction mixture to each active well. Mix gently.
   **Minus-P450 control reactions:** Add 12.5µl of the 4X control reaction mixture to each control well. Mix gently.
3. Pre-incubate the plate at the desired reaction temperature (room temperature or 37°C) for 10 minutes.
   **Note:** See the *P450-Glo™ Assays Technical Bulletin* for a discussion of CYP reaction conditions.
4. Start reactions by adding 25µl of 2X NADPH regeneration system to all wells. 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 the CYP reactions and minus-P450 control reactions.
7. Mix the plate for 10 seconds on an orbital shaker or by gently tapping the plate.
8. Incubate the plate at room temperature for 20 minutes to stabilize the luminescent signal.
9. Record luminescence using a luminometer or CDD camera.
   **Note:** Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Do not use a fluorometer. Do not use filters with the luminometer.

**ORDERING/TECHNICAL INFORMATION:**
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# P450-Glo™ CYP3A4 Assay (Luciferin-IPA)

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## Performing Nonlytic CYP3A4/Luciferin-IPA P450-Glo™ Assays Using Cultured Cells in Monolayers

Reserve some empty wells in the cell culture plates for background measurements. Recommendations for cell culture conditions are given in the *P450-Glo™ Assays Technical Bulletin* #TB325.

1. Treat cells with test compounds. For CYP gene induction studies cells are typically treated with inducers for 24–72 hours. Optimal treatment time should be determined empirically; however, 48 hours is a common starting point. Change medium with test compounds, etc., once daily for the duration of the treatment time.
2. After the experimental treatment, replace culture medium with fresh medium containing 3µM Luciferin-IPA.

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

   **Note:** If inhibitors of basal or induced CYP enzyme activity are being tested, add them at this point with the Luciferin-IPA.
3. To determine background luminescence, add Luciferin-IPA in medium to a set of empty wells (no cells).
4. Incubate plates at 37°C for 30–60 minutes.
5. Transfer 50µl of medium from each well to a 96-well opaque white luminometer plate at room temperature, and add 50µl of Luciferin Detection Reagent to initiate a luminescent reaction (for single-tube luminometers use appropriate luminometer tubes or cuvettes).
6. Incubate the plate at room temperature for 20 minutes, then read luminescence using a luminometer or CCD camera.

   **Note:** Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Do not use a fluorometer. Do not use filters with the luminometer.
7. Calculate net signals by subtracting background luminescence values from test compound-treated and untreated (vehicle control) values.
8. Calculate fold changes by dividing treated values by untreated values.
9. **Optional:** Perform the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570, G7571, G7572 and G7573) to normalize P450-Glo™ Assay values to cell number.

**Instructions for Use:** For complete protocol information, see the *P450-Glo™ Assays Technical Bulletin* #TB325, available online at: **www.promega.com/tbs/** or upon request from Promega.

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# P450-Glo™ CYP3A4 Assay (Luciferin-IPA)

INSTRUCTIONS FOR USE OF PRODUCTS V9001 AND V9002.

Quick PROTOCOL

## Performing Lytic CYP3A4/Luciferin-IPA P450-Glo™ Assays Using Cultured Cells in Monolayers

1. Treat cells with test compounds. For CYP gene induction studies cells are typically treated with inducers for 24–72 hours. Optimal treatment time should be determined empirically; however, 48 hours is a common starting point. Change the medium with test compounds, etc., once daily for the duration of the treatment time.
2. After the experimental treatment replace culture medium with fresh medium containing 3µM Luciferin-IPA.

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

   **Note:** If inhibitors of basal or induced CYP enzyme activity are being tested, add them at this point with the Luciferin-IPA.
3. To determine background luminescence, add Luciferin-IPA in medium to a set of empty wells (no cells).
4. Incubate plates at 37°C for 30–60 minutes.
5. Add an equal volume of Luciferin Detection Reagent to each well, and mix briefly on a multiwell plate shaker or by gently tapping or swirling the plate to form a lysate.
6. **Option 1:** Transfer 50µl of lysate from each well to a 96-well opaque white luminometer plate at room temperature (for single-tube luminometers use appropriate luminometer tubes or cuvettes).

   **Option 2:** Read luminescence directly from cell culture plate. In this case, to avoid luminescent cross-talk between wells, cells must be grown in a white-walled culture plate with clear-bottom wells.
7. Equilibrate the plate at room temperature for 15–20 minutes.

   **Note:** Luminescence from lysates of some, but not all, cell types may decay rapidly, so it might be necessary to read luminescence as soon as possible after adding Luciferin Detection Reagent.
8. Read luminescence using a luminometer or CCD camera.

   **Note:** Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Do not use a fluorometer. Do not use filters with the luminometer.
9. Calculate net signals by subtracting background luminescence values from test compound-treated and untreated (vehicle-control) values.
10. Calculate fold changes by dividing treated net values by untreated net values.

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## Performing Lytic CYP3A4/Luciferin-IPA P450-Glo™ Assays with Suspension Cells

We recommend this protocol for monitoring basal CYP activity and inhibition of basal activity. We do not recommend the use of suspension cells to measure CYP induction.

Use 96-well plates with white walls.

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

1. Prepare a 2X Luciferin-IPA/2X test compound solution in culture medium. Prepare 50µl for each well.
2. Adjust cell concentration to 2 x $10^6$ cells/ml in vendor-recommended serum-free medium.
3. Add 50µl of homogeneous cell suspension to each well.
4. Add 50µl of 2X Luciferin-IPA/2X test compounds to the cell suspension in each well. To control wells without cells, add 50µl of 2X Luciferin-IPA to 50µl of culture medium.
5. Incubate the plate at 37°C, 5% $CO_2$ for 15 minutes for Luciferin-IPA reactions.
6. Add an equal volume (100µl) of Luciferin Detection Reagent to each well. Shake briefly on an orbital shaker.
7. Equilibrate plate at room temperature for 15–20 minutes.
   **Note:** The luminescence with lysates from some, but not all, cell types may decay rapidly, so it might be necessary to read luminescence as soon as possible after adding Luciferin Detection Reagent.
8. Read luminescence.
   **Note:** Luminometer settings will depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. Do not use a fluorometer. Do not use filters with the luminometer.

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