AUTOMATED PROTOCOL

P450-Glo™ CYP450 Assays Automated Protocol

Instructions for Use of Products
V8752, V8762, V8772, V8782, V8792, V8802 and V8812

Revised 2/15
EP026

P450-Glo™ CYP450 Assays Automated Protocol

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 Automated Protocol.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

This document describes automation of the P450-Glo™ CYP450 Assays[a,b]. Specific instructions are provided for a 384-well assay format using a Beckman Coulter Biomek® FX laboratory workstation with a 96-channel head. For information about obtaining the automated method, please see: **www.promega.com/automethods/**

General automation guidelines are provided for adaptation to other liquid handling platforms. For detailed information on the assay chemistry, please refer to the *P450-Glo™ CYP450 Assays Technical Bulletin* #TB325.

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP1A1 Assay	**50ml**	**V8752**

Includes:

- 1 × 350µl Luciferin-CEE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml P450-Glo™ Buffer

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP1B1 Assay	**50ml**	**V8762**

Includes:

- 1 × 350µl Luciferin-CEE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml P450-Glo™ Buffer

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP1A2 Assay	**50ml**	**V8772**

Includes:

- 1 × 1ml Luciferin-ME, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml P450-Glo™ Buffer

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP2C8 Assay	**50ml**	**V8782**

Includes:

- 2 × 750µl Luciferin-ME, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml P450-Glo™ Buffer

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP2C9 Assay	**50ml**	**V8792**

Includes:

- 1 × 1ml Luciferin-H, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml P450-Glo™ Buffer

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP3A4 Assay	**50ml**	**V8802**

Includes:

- 1 × 500µl Luciferin-BE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml P450-Glo™ Buffer

PRODUCT	SIZE	CAT.#
P450-Glo™ CYP3A7 Assay	**50ml**	**V8812**

Includes:

- 2 × 750µl Luciferin-BE, 5mM
- 1 vial Luciferin Detection Reagent (lyophilized)
- 1 × 50ml P450-Glo™ Buffer

Each system contains sufficient reagents for 1,536 assays at 25µl per assay in 384-well plates. To measure cytochrome P450 activity, 25µl of Luciferin Detection Reagent is added to each well, bringing the total volume per well to 50µl. The number of assays per 50ml bottle will increase for multiplate methods.

Storage Conditions: Store all components at –20°C protected from light. The reconstituted Luciferase Detection Reagent can be stored at –20°C for up to 3 months. For convenience, the reconstituted Luciferin Detection Reagent can be stored at room temperature (approximately 23°C) without loss of activity for 24 hours or at 4°C for 1 week. Avoid multiple freeze-thaw cycles of all components. When stored and handled properly, each system is guaranteed for 6 months from the date of purchase.

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3. Before You Begin

Materials to Be Supplied by the User

(Solution compositions are provided in Section 3.A)

- 1M KPO_4 (pH 7.4)
- 2M KPO_4 (pH 7.4) [For CYP3A4 only]
- Distilled, deionized, or nuclease-free water
- Active cytochrome P450 preparation that includes cytochrome P450 reductase (See the *P450-Glo™ CYP450 Assays Technical Bulletin* #TB325 for supplier information.)
- Preparation that lacks cytochrome P450 activity for negative control reactions
- 4X NADPH regenerating system
- Luminescence plate reader (e.g., BMG FLUOstar OPTIMA or equivalent) capable of reading multiwell plates

Note: In this automated protocol, we recommend preparing a 4X NADPH regenerating system and a 2X cytochrome P450/KPO_4/substrate reaction mixture. This is different from the recommentations given in the *P450-Glo™ CYP450 Assays Technical Bulletin* #TB325 because the Biomek® FX will pipet the larger volume of the 2X cytochrome P450/KPO_4/substrate reaction mixture more accurately. The components of the 4X NADPH regenerating system are in excess, so less accurate pipetting of the smaller volume of this solution will have little effect on assay variation.

1M KPO_4 (pH 7.4)

13.94g	potassium phosphate, dibasic, anhydrous (K_2HPO_4)
2.72g	potassium phosphate, monobasic, anhydrous (KH_2PO_4)

Bring the volume to approximately 90ml with deionized water. Adjust to pH 7.4 with KOH or H_3PO_4. Add deionized water to a final volume of 100ml. Use to prepare 2X cytochrome P450/KPO_4/substrate reaction mixtures.

2M KPO_4 (pH 7.4) for CYP3A4

27.87g	potassium phosphate, dibasic, anhydrous (K_2HPO_4)
5.44g	potassium phosphate, monobasic, anhydrous (KH_2PO_4)

Bring the volume to approximately 90ml with deionized water. Adjust to pH 7.4 with KOH or H_3PO_4. Add deionized water to a final volume of 100ml. Use to prepare CYP3A4 4X NADPH regenerating system.

4X NADPH regenerating system

5.2mM	$NADP^+$
13.2mM	glucose-6-phosphate
0.8u/ml	glucose-6-phosphate dehydrogenase
13.2mM	$MgCl_2$

To limit activity, keep the glucose-6-phosphate dehydrogenase separate from the other reaction components until ready to use. Concentrated stocks (e.g., 20X or 100X) can be prepared in advance. Store at –20°C.
Note: Each 384-well plate will require ~4.5ml of 4X NADPH regenerating system.

4X NADPH regenerating system for use with CYP3A4

5.2mM	$NADP^+$
13.2mM	glucose-6-phosphate
0.8u/ml	glucose-6-phosphate dehydrogenase
13.2mM	$MgCl_2$
800mM	KPO_4 (pH 7.4)

To limit activity, keep the glucose-6-phosphate dehydrogenase separate from the other reaction components until ready to use. Concentrated stocks (e.g., 20X or 100X) can be prepared in advance. Store at –20°C.

3.A. Preparing Buffers and Solutions

Table 1. Concentration of Components of the 2X Cytochrome P450/KPO_4/Substrate Reaction Mixture.

Cytochrome P450 Isoform	Amount of Cytochrome P450[1]	KPO_4 Concentration	Substrate Concentration[2]
CYP1A1	0.25pmol	200mM	60µM Luciferin-CEE
CYP1A2	0.25pmol	200mM	200µM Luciferin-ME
CYP1B1	0.5pmol	200mM	40µM Luciferin-CEE
CYP2C8	0.5pmol	100mM	300µM Luciferin-ME
CYP2C9	0.25pmol	50mM	200µM Luciferin-H
CYP3A4	0.5pmol	See table note 3	100µM Luciferin-BE
CYP3A7	0.5pmol	200mM	300µM Luciferin-BE
Liver microsomes	10µg (total protein)	200mM	40–300µM substrate of choice

Concentrations are appropriate for addition of 12.5µl P450/KPO_4/substrate reaction mixture per well. Please refer to the *P450-Glo™ CYP450 Assays Technical Bulletin* #TB325 for additional information.

[1]Recommended amount of cytochrome P450 per 25µl reaction.

[2]The substrate concentration listed is 2 times the apparent K_m for the indicated isoform of cytochrome P450.

[3]For assay with CYP3A4, prepare a 2X cytochrome P450/substrate reaction mixture without KPO_4. Better results are obtained when the KPO_4 is withheld from the 2X reaction mixture and is added at a 4X concentration (800mM) as a component of the 4X NADPH regenerating system (see Section 3.A).

3.B. Preparing Reconstituted Luciferin Detection Reagent

1. Equilibrate the P450-Glo™ Buffer and Luciferin Detection Reagent to room temperature.
2. Transfer the contents of one 50ml bottle of P450-Glo™ Buffer to the amber bottle containing the lyophilized Luciferin Detection Reagent.
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, dispense into aliquots and store at –20°C.

3.C. Preparing 2X Cytochrome P450/KPO_4/Substrate Reaction Mixture

1. Thaw the cytochrome P450 preparation rapidly at 37°C, and place on ice.
2. Thaw the appropriate P450-Glo™ substrate and keep on ice.
3. Prepare the 2X Cytochrome P450/KPO_4/substrate reaction mixture using the concentration of each component listed in Table 1. Prepare enough reaction mixture to dispense 70µl per well in a 96-well PCR plate (~7ml). Keep the mixture on ice until ready to use.
4. For negative control reactions (minus cytochrome P450), prepare a 2X Control/KPO_4/substrate reaction mixture using an equivalent amount of a preparation that lacks cytochrome P450 activity. Prepare enough control reaction mixture to dispense 70µl per well in a 96-well PCR plate (~7ml). Keep the mixture on ice until ready to use.

   Note: This control will provide a measure of background luminescence from the assay system.

4. Automated Processing Requirements for the Biomek® FX Workstation

4.A. Instrument Requirements for the Biomek® FX

The following is a list of parts and their corresponding part numbers required for use of the P450-Glo™ Assays on a Beckman Coulter Biomek® FX instrument. Any single-arm multichannel Biomek® FX is able to run the method. The method can also be adapted for a dual-arm Biomek® FX with at least one multichannel POD.

Part Description	Quantity	Ordering Information
Biomek® FX Bioworks™ Software Version 2.1 (minimum)		Contact Beckman Coulter
96-channel POD	1	Beckman Coulter Part# 719368
Minimum Number of Labware Positions by 1 POD	11	Contact Beckman Coulter
Tip Loader ALP	1	Beckman Coulter Part# 719356
Orbital Shaker ALP	1	Beckman Coulter Part# 379448

4.B. Labware Requirements for the Biomek® FX

Part Description	Quantity	Ordering Information
Costar® 96-well, flat-bottom white polystyrene microplate	1	Corning Cat.# 3912
96-well, full-skirt polypropylene plate	3	Greiner Cat.# 652270
384-well, 100µl white flat-bottom uniplate	1	Whatman Cat.# 7701-3100
Biomek® AP96 P20 Tips Sterile, Barrier	5 racks	Beckman Coulter Cat.# 717256
Flat Thermal Adapter (used only for 37°C incubation method)	1	ACME Automation Cat.# C5077

4.C. Initial Deck Layout for the Biomek® FX

This is an example of the Biomek® FX deck layout for 384-well, automated P450-Glo™ Assays. **Your specific deck layout may be different depending on your Biomek® FX configuration.**

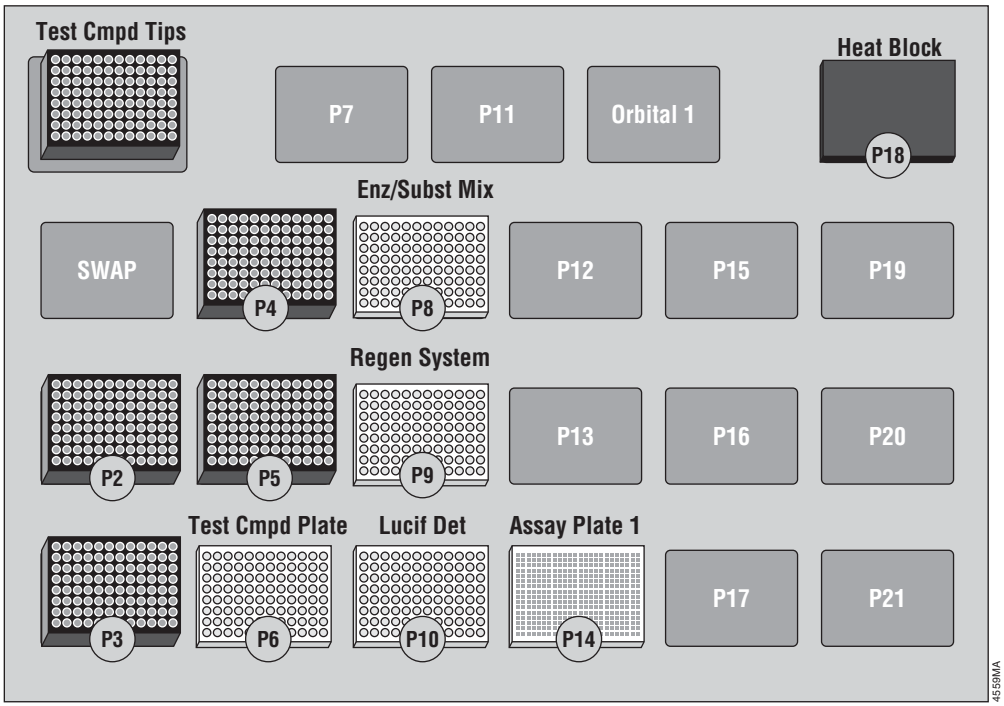

Figure 1. Biomek® FX initial deck configuration.

ALP Name	Equipment
Tip loader	Biomek® AP96 P20 Barrier Tips
P1	Empty: Tip box swap spot
P2–P5	Biomek® AP96 P20 Barrier Tips
P6	Costar® 96-well microplate containing 45µl per well test compound or water
P7	Empty
P8	96-well full-skirt plate containing 70µl per well 2X cytochrome P450/KPO_4/substrate reaction mixture
P9	96-well full-skirt plate containing 45µl per well 4X NADPH regenerating system
P10	96-well full-skirt plate containing 125µl per well Luciferin Detection Reagent
P11–P13	Empty
P14	Empty 384-well flat-bottom Uniplate
P15–P17, P19–P21	Empty
P18	Heating/Cooling ALP with 384-well Flat Thermal Adapter (used only during 37°C incubation method)
Orbital 1	Orbital Shaker ALP

4.D. Biomek® FX Specific Pre-Run Recommendations

The Biomek® FX allows users the flexibility to configure the robot's deck according to need. This flexibility makes it likely that the deck used for writing a Biomek® FX method will differ from an end-user's deck. Therefore, it is generally necessary to map an imported method onto an end-user's deck configuration. To map an imported method onto your deck, please follow the instructions provided in the document: *Biomek® FX Deck Mapping* (**www.promega.com/automethods/beckman/biomekfx/default.asp**).

Prior to the first run, ensure that the Biomek® FX deck has been properly framed. Failure to do so may result in bending of tips during the method.

The P450-Glo™ Assays may be performed at room temperature or at 37°C. If you are performing your assay at 37°C, turn on the water bath connected to the Heating/Cooling ALP, and set it to the appropriate temperature to achieve a 37°C incubation temperature within the wells of the assay plate. Place a 384-well Flat Thermal Adapter onto the Heating/Cooling ALP.

5. Description of the P450-Glo® Assay Automated Protocol

This overview describes the general liquid handling steps required for use of the P450-Glo™ Assays in 384-well format. **Note:** You will be prompted to enter an incubation time when setting up the method.

For information about adaptation to instruments other than the Biomek® FX, please refer to Section 6.

5.A. Adding Test Compound/Cytochrome P450 Substrate Mix

Note: The volume of organic solvent should be kept to a minimum to avoid potential effects on cytochrome P450 activities. For example, DMSO is a known CYP3A4 inhibitor.

1. **Test Compound/Water Transfer.** Test compound (6.25µl/well) is transferred to the 384-well assay plate. If assays are being performed without substrate, 6.25µl of water is transferred to each well.
2. **2X Cytochrome P450/Substrate Mix Transfer.** 2X Cytochrome P450/substrate reaction mixture (12.5µl), or 2X control/substrate reaction mixture (12.5µl) is transferred to each well of the 384-well assay plate. Wells designated as water blanks receive 12.5µl of water.
3. **Assay Plate Mix.** The assay plate is transferred to the orbital shaker and mixed for 20 seconds at 1,200rpm. The plate is then transferred back to its original position. In the 37°C incubation method the assay plate is transferred to the Heating/Cooling ALP with the 384-well Flat Thermal Adapter.
4. **Assay Plate Incubation.** The plate is incubated at room temperature (or 37°C) for 10 minutes. In the 37°C incubation method, the assay plate is transferred back to its original position at the end of the incubation period.

5.B. Adding 4X NADPH Regenerating System

1. **4X NADPH Regenerating System Transfer.** 4X NADPH regenerating system (6.25µl/well) is added to the 384-well assay plate.

   Note: For reactions with CYP3A4, we recommend that the KPO_4 be added as a component of the 4X NADPH regenerating system.
2. **Assay Plate Mix.** The assay plate is transferred to the orbital shaker and mixed for 20 seconds at 1,200rpm. The plate is then transferred back to its original position. In the 37°C incubation method the assay plate is transferred to the Heating/Cooling ALP with 384-well Flat Thermal Adapter.
3. **Assay Plate Incubation.** The plate is incubated at the same temperature used in Section 5.A Step 4. Refer to the *P450-Glo™ CYP450 Assays Technical Bulletin* #TB325, Table 2, for general incubation guidelines. In the 37°C incubation method, the assay plate is transferred back to its original position at the end of the incubation period.

5.C. Adding Luciferin Detection Reagent

1. **Luciferin Detection Reagent Transfer.** Luciferin Detection Reagent (2 × 12.5µl) is transferred to each well of the 384-well assay plate.
2. **Assay Plate Mix.** The assay plate is transferrred to the orbital shaker and shaken for 10 seconds at 1,000rpm. After completion of the mix, the plate is transferred back to its original position.

5.D. Measuring Luminescence

1. **Assay Plate Incubation.** Incubate the assay plate at room temperature for 20 minutes to stabilize the luminescent signal.
2. Manually assay samples using a luminescence plate reader.

6. General Guidelines for Adaptation to Alternative Robotic Platforms

Care should be taken to avoid contamination with solutions containing luciferin. We recommend using barrier tips for all reagent transfers.

The cytochrome P450/KPO_4/substrate reaction mixtures need to be kept in suspension during transfer to the assay plate. Frequent mixing is critical and should be performed before all liquid additions.

If using a 384-well plate on a plate shaker, optimize the shaker speed to give best mixing while avoiding spillage.

The addition of small volumes to assay plates can be challenging. Whenever possible, small volume tips should be used to increase reproducibility of reagent addition. For the Biomek® FX automated protocol, we optimized the volumes and concentrations of the cytochrome P450/KPO_4/substrate reaction mixture and the NADPH regenerating system to ensure greater accuracy and consistency of automated pipetting.

7. Summary of Changes

The following changes were made to the 2/15 revision of this document:

1. The patent/license statements were updated.
2. The document design was updated.

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

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

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