P450-Glo™ CYP2C19 and CYP2D6 Assays and Screening Systems: The Method of Choice for In Vitro P450 Assays

JAMES J. CALI, PH.D., DONGPING MA, M.S., MARY SOBOL, M.S., TROY GOOD, PH.D. AND DAVID LIU, PH.D., PROMEGA CORPORATION

The human cytochrome P450 enzymes CYP2C19 and CYP2D6 contribute substantially to the metabolism of therapeutic drugs. The P450-Glo™ Assays for these enzymes enable researchers to assay activity and screen for P450 inhibition using a bioluminescent assay that minimizes background noise and is amenable to high-throughput applications.

Introduction

Cytochrome P450 enzymes (P450s) catalyze the oxidative metabolism of a vast array of hydrophobic chemicals including therapeutic drugs (1). The inhibition of cytochrome P450s by drugs is an important consideration in drug discovery; it can alter drug disposition and cause adverse drug-drug interactions (2). For example, if a first drug inhibits the metabolism of a second co-administered drug, the second drug may accumulate to a toxic level. There are 57 P450 genes in humans (3), and enzymes encoded by only five of them, CYP1A2, 2C9, 2C19, 2D6 and 3A4, account for 80–90% of cytochrome P450-dependent drug metabolism (4).

The P450-Glo™ Assays enable researchers to move beyond the limiting features of traditional cytochrome P450 assay methods.

P450-Glo™ Assay Principle

In small-molecule drug discovery, in vitro test systems are commonly employed to detect P450 inhibition by candidate compounds. For this purpose we developed the P450-Glo™ Assays and Screening Systems[a,b] in which luminogenic P450 substrates are used as probes for P450 activity in recombinant P450 preparations or liver microsomes. Drugs that inhibit these marker activities are identified as P450 inhibitors or substrates that compete with the probe for the P450 active site. The probes are derivatives of the firefly luciferase substrate D-luciferin; however, they are inactive in the light-generating reaction with luciferase.

For P450-Glo™ Assays an initial reaction is performed in which a cytochrome P450 converts a luminogenic substrate to a luciferin product (Figure 1). At the end of the P450 reaction, a Luciferin Detection Reagent is added that contains luciferase and ATP. This reagent simultaneously stops the P450 reaction and initiates a luciferase reaction. The amount of light generated is directly proportional to the amount of luciferin product produced by P450 in the first reaction. We described ssays for CYP1A2, 2C9 and 3A4 previously (5–7), and here we describe P450-Glo™ CYP2C19 and CYP2D6 Assays.

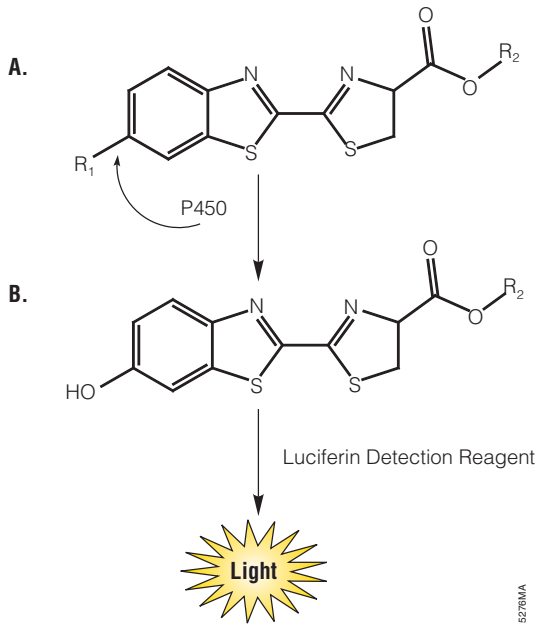

Figure 1. P450-Glo™ Assay general reaction scheme. Compound A represents the P450-Glo™ Substrates and compound B the luminogenic reaction product that generates light with P450-Glo™ Luciferin Detection Reagent. Cytochrome P450 selectivity for P450-Glo™ Substrates is dependent on R_1 and R_2. CYP2C19 substrate: R_1=H, R_2=C_2H_4OH; CYP2D6 substrate: R_1=CH_3O, R_2= C_2H_4OH.

P450-Glo™ Substrate Chemistry

The P450 enzyme selectivity of P450-Glo™ luminogenic substrates depends on the nature of the modifications on D-luciferin, designated R_1 and R_2 in Figure 1. Modifications at both sites substantially block reactivity with luciferase. Oxidation by many P450 enzymes at the R_1 site has been observed. For each isoform, oxidation at the R_1 site by a P450 introduces the hydroxyl group of D-luciferin required for luciferase activity. For some P450s, including CYP1A2, 2C9 and 3A4, an R_1 modification is sufficient; however, for CYP2C19 and 2D6 it is also necessary to modify R_2. The P450-Glo™ CYP2C19 and 2D6 substrates are Luciferin-H EGE and Luciferin-ME EGE, respectively. The EGE stands for ethylene glycol ester and refers to the ethylene glycol unit in ester linkage at the R_2 site. R_1 in Luciferin-H EGE is hydrogen, and in Luciferin-ME, EGE is a methoxy group

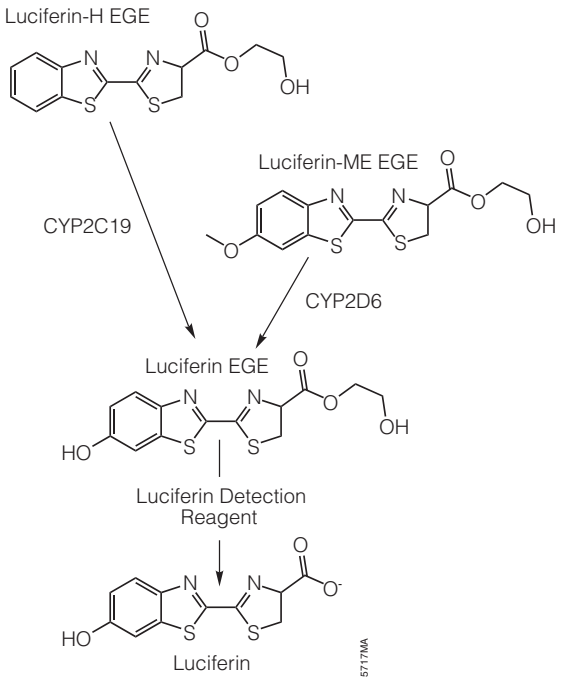

Figure 2. P450-Glo™ Assay CYP2C19 and CYP2D6 reaction schemes. Luciferin-H EGE and Luciferin-ME EGE are converted to luciferin EGE by CYP2C19 and CYP2D6, respectively. Upon addition of Luciferin Detection Reagent, the Luciferin EGE is rapidly de-esterified to luciferin, which reacts with luciferase to produce light.

(Figure 2). Aromatic hydroxylation by CYP2C19 and demethylation by CYP2D6 introduce the essential hydroxyl of D-luciferin at R_1, so the product of both reactions is the luciferin ester. During the first few minutes after adding the Luciferin Detection Reagent this inactive intermediate is de-esterified to completion by an esterase activity in the reagent.

The role of the ester group in the activity of CYP2D6 toward the R_1 methoxy derivative of D-luciferin may be explained in part by the neutralization of an acidic group in the substrate due to addition of the ethylene glycol ester. Three dimensional models of CYP2D6 place two acidic residues (Asp 301 and Glu 216) with their negative charges in the active site (8). An esterified luciferin would be neutral at R_2, while a nonesterified form would carry a negative charge and be expected to be repelled from the active site of CYP2D6 by the acidic residues. Indeed, CYP2D6 substrates are typically neutral or cationic (9,10). CYP2C19 activity toward the R_1 deoxy derivative of D-luciferin is also enabled by neutralization of the carboxylic acid with the addition of an ester group at R_2 (Luciferin-H EGE). This is consistent with this enzyme's preference for neutral over acidic compounds (11). Increased hydrophobicity of luciferin esters over the carboxylic forms might also improve efficiency of the CYP2C19 and 2D6 reactions because there is generally a positive correlation between hydrophobicity and affinity for P450 active sites (12).

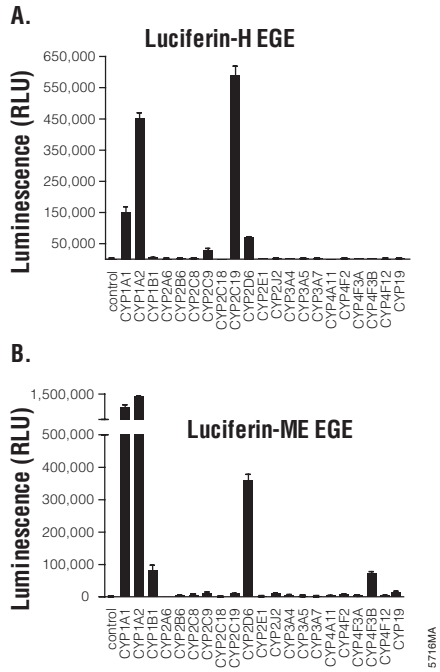

Figure 3. Cytochrome P450 enzyme selectivity for Luciferin-H EGE and Luciferin-ME EGE. Twenty one human cytochrome P450 isoforms were assayed with Luciferin-H EGE (**Panel A**) or Luciferin-ME EGE (**Panel B**). The cytochrome P450s were recombinant enzymes expressed in insect cells and prepared as microsomal fractions. Control reactions used insect cell microsomal fractions devoid of cytochrome P450 activity. One picomole of each cytchrome P450 was assayed for 30 minutes at 37°C in a 50µl volume with KPO_4 buffer (pH 7.4) and an NADPH regeneration system. At the end of the 30-minute reactions Luciferin Detection Reagent was added, and luminescence was recorded on a plate-reading luminometer.

Luciferin-H EGE and Luciferin-ME EGE were screened against a panel of recombinant human P450s and a control sample with no P450 activity using the P450-Glo™ method. Robust activities of CYP2C19 with Luciferin-H EGE and of CYP2D6 with Luciferin-ME EGE were observed (Figure 3). Some other P450s also showed activity toward these substrates, CYP1A1 and 1A2 being the most significant. Luciferin-H EGE and Luciferin-ME EGE could be used for P450-Glo™ Assays of CYP1A1 and 1A2; however, the preferred substrates are Luciferin-CEE, the chloroethyl ether of D-luciferin for CYP1A1 and Luciferin-ME, the methyl ether of luciferin for CYP1A2. The patterns of P450 cross-reactivity seen with Luciferin-H EGE and Luciferin-ME EGE will likely preclude a straight forward application in assays of liver microsomes.

The reactions of CYP2C19 with Luciferin-H EGE and CYP2D6 with Luciferin-ME EGE showed saturation kinetics. A K_m of 10µM and K_{cat} at 3.8(min^{-1}) for the CYP2C19 reaction and a K_m of 30µM and K_{cat} at 7.1min^{-1} for the CYP2D6 reaction were observed (Figure 4). For the K_{cat} measurement the signal output of P450-Glo™ Assays, which is measured in

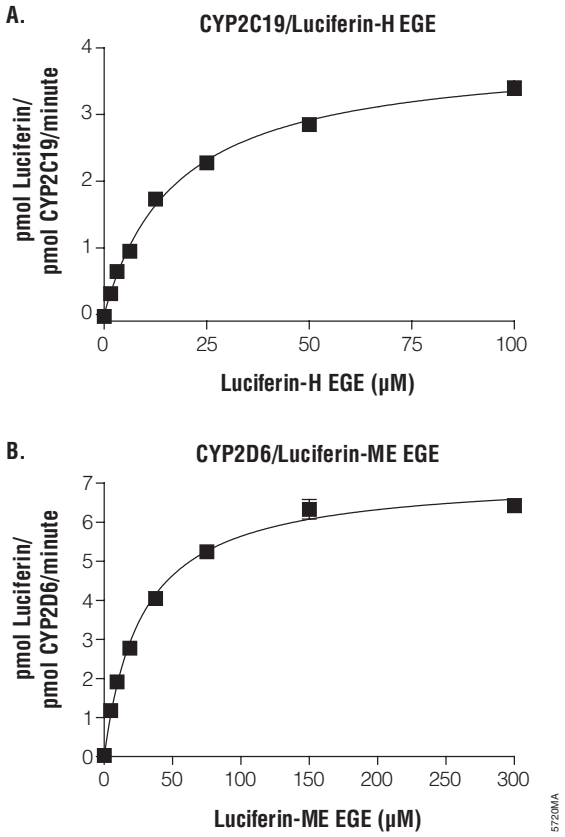

Figure 4. Substrate dose dependence of CYP2C19/Luciferin-H EGE and CYP2D6/Luciferin-ME EGE. CYP2C19 and 2D6 reactions were performed at a range of Luciferin-H EGE and Luciferin-ME EGE concentrations, respectively using 0.25pmol of CYP2C19 in 50mM KPO_4 (pH 7.4) or 0.25pmol of CYP2D6 in 100mM KPO_4 (pH 7.4). Reactions were incubated for 20 or 30 minutes, respectively, in the presence of an NADPH regeneration system. Assays were stopped and luminescence initiated by adding an equal volume of Luciferin Detection Reagent. Each reaction was then adjusted to the highest substrate concentration in the range so that the effect of unmetabolized substrate on luciferase activity would be constant across the range. Luminescence (RLU) was read on a GloMax™ 96 Microplate Luminometer (Cat.# E6501). RLU values were converted to luciferin concentrations by interpolation from a luciferin standard curve read in parallel with the cytochrome P450 reactions. Luciferin concentrations were then used to calculate activities in terms of picomoles luciferin produced per picomole cytochrome P450 per minute.

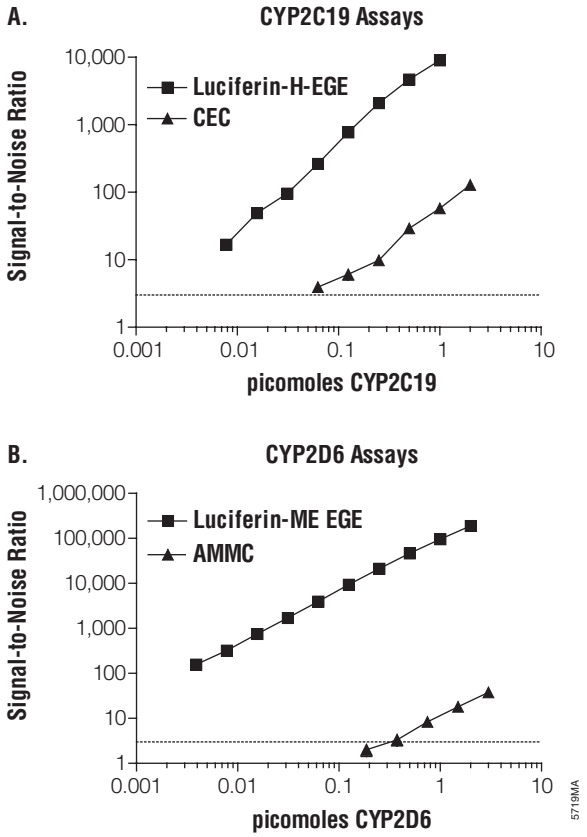

Figure 5. P450-Glo™ Assay sensitivity. Cytochrome P450 assays were performed at 37°C with recombinant cytochrome P450 microsome fractions from an insect cell expression system. P450-Glo™ Assays were performed as described in Technical Bulletins #TB325 and #TB340, and fluorescent assays as described by BD/Gentest (CEC = 3-Cyano-7-ethoxycoumarin, AMMC = 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin). Relative luminescent or fluorescent measurements from experimental samples and minus-P450 (background) controls were made with a plate-reading luminometer or fluorometer. To calculate signal-to-noise ratios, the average background measurements were subtracted from experimental sample measurements to give signals, and signals were divided by the noise (noise = the standard deviation of the background; 13).

relative light units (RLU) on a luminometer, was converted to D-luciferin concentrations by interpolation from a luminescent D-luciferin standard curve (13).

Low Background Allows Greater Assay Sensitivity

The sensitivities of the reactions of CYP2C19 with Luciferin-H EGE and CYP2D6 with Luciferin-ME EGE were examined by varying the amount of cytochrome P450 enzyme in reactions at the K_m concentrations of the substrates (Figure 5). When the data are expressed as signal-to-noise ratios and compared to assays with commonly used fluorescent cytochrome P450 substrates, the P450-Glo™ Assays are clearly more sensitive. This sensitivity is due in part to low background signals of P450-Glo™ Assays.

The increase in cytochrome P450-dependent luminescence from the CYP2C19 reaction with Luciferin-H EGE and the CYP2D6 reaction with Luciferin-ME EGE was monitored over time at 37°C and room temperature (approximately 22°C; Figure 6). Luminescence increased linearly at both temperatures for at least 45 minutes, but linearity persisted somewhat longer at room temperature, probably because the enzymes are more stable at the lower temperature.

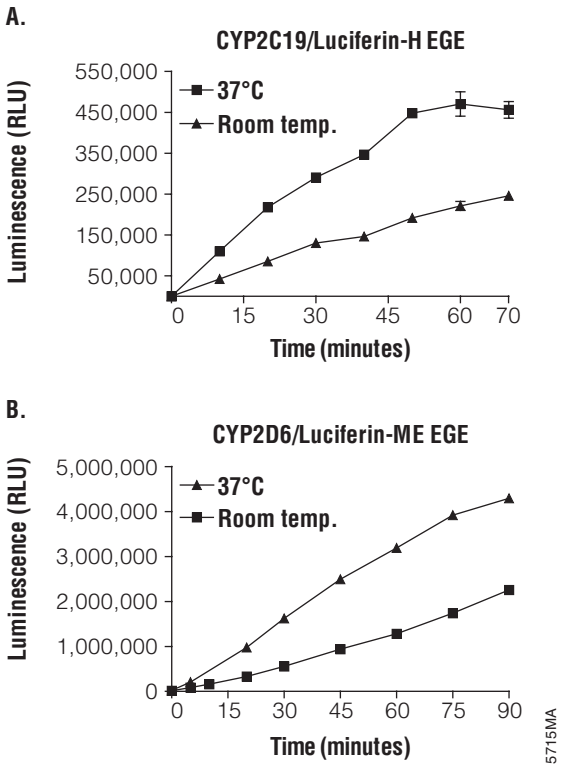

Figure 6. Incubation time and temperature. 0.25pmol CYP2C19 with 10µM Luciferin-H EGE or 0.50pmol CYP2D6 with 50µM Luciferin-ME EGE were assayed as described in the legend for Figure 4. The P450-Glo™ Assays were incubated at room temperature or 37°C for up to 90 minutes before adding the reconstituted Luciferin Detection Reagent.

Obtain Accurate IC_{50} Data

The P450-Glo™ CYP2C19/Luciferin-H EGE and CYP2D6/Luciferin-ME EGE Assays were used to measure inhibition of CYP2C19 and CYP2D6 by known inhibitors of the respective enzymes (Figure 7). Reactions were performed at a range of inhibitor concentrations with 0.25pmol of each enzyme at the respective K_m substrate concentrations for 20 minutes (CYP2C19) or 30 minutes (CYP2D6) at 37°C. Dose-dependent inhibition was observed for each inhibitor with IC_{50} values similar to published values using other probe substrates (14–17).

Summary

The bioluminescent approach of the P450-Glo™ Assay has certain fundamental advantages over fluorescent methods. For example, the excitation light used for fluorescence measurements can create high background signals that limit assay sensitivity. In contrast bioluminescence does not use an excitation light because luciferase excites the photon emitter enzymatically. Background signals are therefore lower and

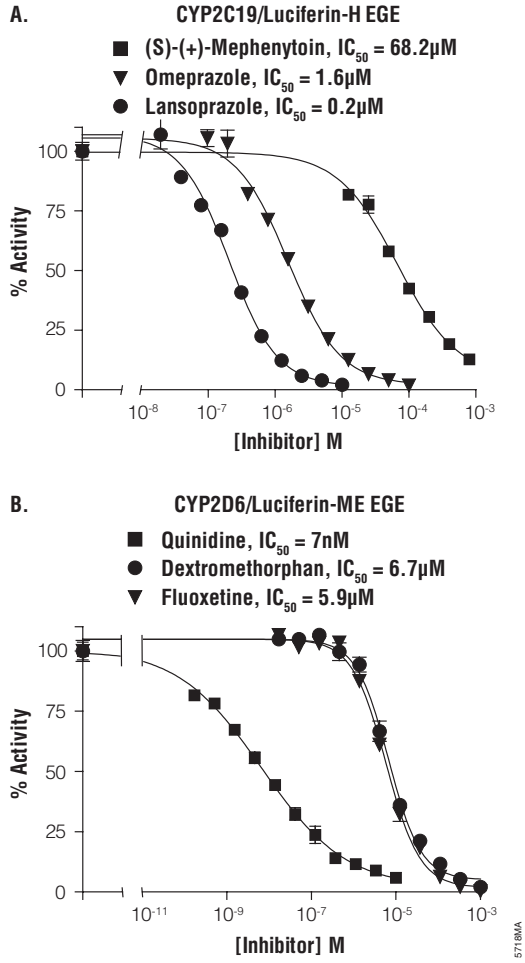

Figure 7. Using the P450-Glo™ Assay to measure P450 Inhibition. Recombinant cytochrome P450s were assayed at 37ºC at the K_m concentrations of their respective substrates and the indicated concentrations of inhibitors. Curve fits and IC_{50} calculations were performed with the GraphPad Prism® program.

assays more sensitive. Also, misleading results due to overlap between fluorescent probe substrates and fluorescent analytes or other assay components (e.g., NADPH) are eliminated with the bioluminescent approach. By adapting these fundamental advantages of bioluminescence, the P450-Glo™ Assays enable researchers to move beyond many of the limiting features of traditional cytochrome P450 assay methods. Assay robustness and ease of use, exquisite sensitivity, adaptability to automation and low false-positive rates are a few of the reasons why P450-Glo™ Assays are the method of choice for in vitro cytochrome P450 assays. ■

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Protocols

P450-Glo™ Screening Systems Technical Bulletin #TB340 (www.promega.com/tbs/tb340/tb340.html)

P450-Glo™ Assays Technical Bulletin #TB325 (www.promega.com/tbs/tb325/tb325.html

Ordering Information

Product	Size	Cat.#
P450-Glo™ CYP2D6 Assay	10ml	V8891
	5ml	V8892
P450-Glo™ CYP2C19 Assay	10ml	V8881
	50ml	V8882
P450-Glo™ CYP2D6 Screening System	1,000 assays	V9890
P450-Glo™ CYP2C19 Screening System	1,000 assays	V9880

[a]U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents pending.

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

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Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711-5399 *USA*
Tel: 608-274-4330
Fax: 608-277-2516
Toll-Free: 800-356-9526
Toll-Free Fax: 800-356-1970
Internet: www.promega.com

Promega Biosciences, Inc.
A Division of Promega Corporation
San Luis Obispo, California

***Australia,* Sydney**
Tel: 02 9565 1100
Fax: 02 9550 4454
Freecall: 1800 225 123
Freefax: 1800 626 017
E-mail: aus_custserv@au.promega.com

***China,* Beijing**
Tel: (86) 10 5825 6268
Fax: (86) 10 5825 6160
Freefax: 18 0062 6017
E-mail: promega@promega.com.cn

***France,* Lyon**
Tel: 04 37 22 50 00
Fax: 04 37 22 50 10
Numero Vert: 0 800 48 79 99
E-mail: fr_custserv@fr.promega.com

***Germany/Austria,* Mannheim**
Tel: (+49) (0) 621 8501 0
Fax: (+49) (0) 621 8501 222
Free Phone: 00800 77663422
Free Fax 00800 77663423
E-mail: de_custserv@de.promega.com

***Italy,* Milan**
Tel: 02 54 05 01 94
Fax: 02 55 18 56 64
Numero Verde: 800 69 18 18
E-mail: it_custserv@it.promega.com

***Japan,* Tokyo**
Tel: 03 3669 7981
Fax: 03 3669 7982
E-mail: jptechserv@jp.promega.com

***Latin America Region,* Brazil**
Tel/Fax: (55 31) 3262 2915
E-mail: latinoam@promega.com

***Belgium/Luxembourg/ The Netherlands,* Leiden**
Tel: (+31) (0) 71 5324244
Fax: (+31) (0) 71 5324907
Free Tel BE: 0800 18098
Free Fax BE: 0800 16971
Free Tel NL: 0800 0221910
Free Fax NL: 0800 0226545
E-mail: bnl_custserv@nl.promega.com

***Pacific Asia Region,* Singapore**
Tel: 65 6725 9915
Fax: 65 6725 9889
E-mail: sg_custserv@promega.com

***Spain,* Madrid**
Tel: 902 538 200
Fax: 902 538 300
E-mail: esp_custserv@promega.com

***Switzerland,* Wallisellen**
Tel: 044 878 90 00
Fax: 044 878 90 10
Technical Service: 044 878 90 20
E-mail: catalys_custserv@promega.com

***United Kingdom,* Southampton**
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Fax: 023 8076 7014
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