HUMAN PREGNANE X RECEPTOR (PXR, NR1I2) ACTIVATION ASSAY SYSTEM

Technical Manual for 96-Well Format Assay
Catalogue #s DPX2-ACTIV & DPX2-METAB

Puracyp, Inc.
5900 Sea Lion Place
Suite 130
Carlsbad, CA 92010
760-929-9508
Fax: 760-929-9834
www.puracyp.com

HUMAN PXR NUCLEAR RECEPTOR ACTIVATION ASSAY, OPTIONAL ASSESSMENT OF CELL VIABILITY & CYP3A METABOLISM

This technical manual is available in its most current version at: www.puracyp.com. Please contact Puracyp if you have questions concerning the use of the assay system. 760-929-9508 ext. 106 or 113; Email: sales@puracyp.com

I. DESCRIPTION

Puracyp's nuclear receptor activation kits incorporate mycoplasma-free, stably-transfected cells derived from hepatoma cell lines that have been treated with a cell-arrest agent to prevent further proliferation. Exposure to the cell-arrest agent ensures that the cells in the kits can only be used for a single assay. Enough cells are provided in a vial to seed one 96-well plate. The kit also includes optimized cell culture medium and cell dosing medium. The primary application of this assay kit is to identify agents with the ability to activate human PXR. In addition, assessment of CYP450 enzyme activity can be determined.

Puracyp's *Human PXR Activation Assay System* utilizes DPX2™ cells constructed with a proprietary process for producing stably-transfected cell lines. DPX2™ cells harbor the human PXR gene (NR1I2) and a luciferase reporter gene linked to two promoters identified in the human CYP3A4 gene, namely XREM and PXRE. The DPX2™ cells included in the assay kit remain highly viable upon thawing, and can be immediately dispensed into 96-well plates. There is no need for intermediate spin-and-wash steps. However, determining viability and cell titer is recommended. A list of references describing the use of Puracyp's DPX2™ cells and our other stable cell lines is given in this manual.

In a typical assay, DPX2™ cells are first dispensed into the wells of the assay plate, and then placed in a CO_2 incubator at 37°C to equilibrate overnight. The following day, the cells are treated with user's test compounds and the appropriate positive and negative controls. Since the DPX2™ cells included with the kit are non-proliferative, test treatment should take place within 24 h of plate seeding. Once treated, the cells are returned to the CO_2 incubator at 37°C for 24 h (hPXR activation only) or 48 h (hPXR activation plus CYP3A metabolism). The dosing medium is then discarded, cell viability can be assessed and luciferase activity then measured using a luminescent assay such as Promega's ONE-Glo™. The luminescence light intensity is directly proportional to the extent of PXR activation and accompanying gene transcription in the DPX2™ cells. **It should be noted that the assay described in this manual has been optimized using Promega reagents.**

II. ASSAY KIT COMPONENTS & STORAGE CONDITIONS

Puracyp's *Human PXR Activation Assay System* (Products DPX2™-Activ and DPX2™-Metab) contains materials to perform reporter assays in a 96 well plate format. The kits are constructed so that 32 reporter assays can be performed in triplicate. Individual aliquots of DPX2™ cells and media are intended for a single-use. Once DPX2™ cells have been thawed, they can neither be refrozen nor cultured for future use. Therefore, if an entire 96-well plate is not seeded, the remaining cells should be discarded.

Assay kits are shipped on dry ice for shipments within US. For International shipments, the cells would be shipped in a liquid nitrogen shipper. **If the DPX2™ cells will not be used immediately, they require storage in liquid nitrogen.** The other kit components can be stored at -20° C for up to 4 months. Individual expiration dates are provided with each kit.

KIT COMPONENT	*AMOUNT*	*STORAGE TEMPERATURE*
Tube **A** - DPX2™ Cells (DPX2-ACTIV)	4 million x 1 vial	Liquid Nitrogen
Tube **A** - DPX2™ Cells (DPX2-METAB)	6 million x 1 vial	Liquid Nitrogen
Tube **C** - Culture Media	1 x 10 mL	-20° C
Tube **D** - Dosing Media	1 x 35 mL	-20° C
Tube **E** –CTF Buffer	1 x 10 mL	-20° C

III. MATERIALS TO BE SUPPLIED BY USER

The following materials are required by the end-user and should be available prior to starting the assay procedure.

FOR RECEIPT OF CELLS

- Cell culture-rated hood
- Humidified 5% CO_2 /37°C incubator suitable for mammalian cell culture
- 37° C water bath
- Sterile multi-channel media basins or reservoirs
- 70% ethanol or isopropyl alcohol mixture
- Multi-channel (8- or 12-channel) pipette & sterile tips
- For DPX2-ACTIV kits, Sterile 96-well Corning® BioCoat® Collagen I 96-well White/Clear Flat Bottom TC-treated Microplate, with Lid. (Cat # **354650** or 35650)
- For DPX2-METAB kits, Sterile 96-well tissue culture treated plate with lid (Corning® Cat # **3610**)

FOR DAY 1

- Cell culture-rated hood
- Humidified 5% CO_2 /37°C incubator
- 37°C water bath
- Sterile 1.5 ml tubes for DMSO dilutions
- Sterile 96 deep well plate (2 ml/well capacity, such as Eppendorf Cat.# 9400002001) to make test compound dilutions in media

- DMSO (ultra pure!) or other solvent for use as a test compound diluent and as the negative control
- Multi-channel (8 or 12) pipette & sterile tips
- Rifampicin

FOR DAY 2 (Receptor Activation, DPX2-ACTIV)

Microplate reader with capacity to read luminescence and fluorescence if also determining cell viability with a fluorescence assay, such as Promega's Cell-Titer Fluor. The luminometer portion of the instrument needs to be able to accumulate luminescence RLUs over a 5 sec/well read. Instruments that average the counts per second will produce low counts resulting in low induction and increased well-to-well variability.

- Promega's ONE-Glo™ (cat. # E6110) or other detection reagent for luminescent reagent (hPXR activation)
- As an option, Promega's CellTiter-Fluor™ (cat. # G608A-C) or other fluorescence assay used to detect cell viability .
- 2 x Sterile Media troughs
- 15 ml sterile conical centrifuge tube
- Multi-channel pipette & sterile tips

FOR DAY 3 (*P450 Metabolism & *Receptor Activation, DPX2-METAB)

- **White** 96-well plate (non-sterile) to read luminescence
- Multi-mode microplate reader with capacity to read luminescence and fluorescence. The luminometer portion of the instrument needs to be able to accumulate luminescence RLUs over a 5 sec/well read. Instruments that average the counts per second will produce low counts resulting in low induction and increased well-to-well variability.
- Promega's ONE-Glo™ (cat. # E6110) or other detection reagent for detecting luminescence (hPXR activation
- As an option, Promega's CellTiter-Fluor™ (cat. # G608A) or other fluorescence assay used to detect cell viability. Promega's P450-Glo™ (cat. # V9001) or other labelled substrate for detecting CYP3A Metabolism
- 4 x Sterile Media troughs
- Multi-channel pipette & sterile tips
- 2 x 15 ml sterile conical centrifuge tube
- Sterile Phosphate buffered saline (PBS)

IV. ASSAY PROCEDURE

NOTE: It is advisable to review the following section before proceeding with the assay

Puracyp's *Human PXR Activation Assay* incorporates one overnight and one 24 h or 48 h incubation. *Steps 1-11* are performed upon receipt of cells, and require about **30 min** to complete. *Steps 12-21* are performed on Day 1, and need **1.5 - 4.5 h** to complete, depending on the number of dilutions and compounds to be tested. *Steps 22-32* are performed on Day 2 (DPX2-ACTIV) and *Steps 33-49* are performed Day 3 (DPX2-METAB), and require **1.5-3 h** to perform.

RECEIPT OF CELLS: ALL STEPS MUST BE PERFORMED USING PROPER ASEPTIC TECHNIQUE

1. Wipe down tissue culture hood with 70% alcohol solution.
2. Remove a tissue culture 96-well plate such as Corning 3610 plate, and place inside tissue culture hood.
3. Place sterile media trough or basin in hood.
4. Prepare multi-channel pipette with sterile tips.
5. Place Tube C from kit directly into a 37° C water bath until medium has thawed and warmed to 37°C, and transfer 10 ml into a sterile trough.
6. Place Tube A containing the DPX2™ cells into a 37°C water bath for 1-2 min, or until cell suspension has thawed. **Do Not Overheat**
7. Sterilize outside of Tube A containing the cells with 70% alcohol solution. Using a 2 ml pipette, aspirate the cell mixture up and down twice to mix, then transfer cells to the sterile trough containing 10 ml of medium from Tube C.
8. Remove 2 ml of the cell-medium mixture, and use it to rinse out Tube A. Transfer contents back to the media trough. It is recommended that the cells be counted to ensure there are 4 (DPX2-ACTIV) or 6 million (DPX2-METAB) viable cells/vial (Cell number depends on which kit is purchased, DPX2-ACTIV or DPX2-METAB).
9. Uniformly mix cells with medium by pipetting up and down at least 10X with a 10 ml pipette.
10. Using the multi-channel pipette, transfer 100 µl of cell medium suspension from the trough to the individual wells of 96-well plate. If a re-pipettor is not used during the seeding process, ensure that the cells remain suspended in media by agitating the trough between seeding of each row.
11. Place seeded plate into humidified 5% CO_2 /37°C incubator overnight.

DAY 1

12. Prepare 20 mM stock of rifampicin in DMSO.
13. Place Tube D from kit at room temperature, and allow to thaw.
14. Wipe down tissue culture hood with 70% alcohol solution.

THE FOLLOWING STEPS ARE PERFORMED IN A STERILE HOOD

15. Prepare positive control (rifampicin) dilutions as suggested and described in **Appendix 1.**
16. Prepare Test Compound dilutions as suggested and described in **Appendix 2** if triplicate determinations are planned. Prepare the vehicle control (0.1% DMSO) by adding 1 μl of DMSO to 1 ml of medium from Tube D.
17. Remove 96-well plate containing the cells from the incubator and place into the hood.
18. Prepare the plate for test agent addition by demarcating on the lid those wells that will receive test articles, positive controls, vehicle control, and medium control.
19. Carefully remove the liquid from each well using a multi-channel pipette. *Avoid touching the cells with the pipette tip but ensure that all media is removed; tilting the plate will aid this*.
20. Gently add 100 μl of medium containing test agent, positive control or vehicle control to the plate wells. *Medium addition is performed by gently pipetting the liquid against the well walls rather than directly onto the cells attached to the well bottom.*
21. Return the plate to the incubator, and continue to incubate at 37°C for 24 h or 48 h[A].

[A]Compounds other than RIF may be less stable in culture at 37°C, and may thus require replenishment after 24 h. In those instances, the user should repeat Day 1 steps #12-21 again on Day 2.

DAY 2 PXR Activation, DPX2-ACTIV (If using Promega's Cell-Titer Fluor and ONE-Glo™)

22. Allow Tube E and detection reagents to reach room temperature.
23. Prepare the ONE-Glo™ Assay reagents and invert the bottle 5X to mix.
24. Transfer 10 ml from Tube E to a sterile 15 ml conical tube, and add 5 μl from CellTiter-Fluor™. Mix by inverting tube.
25. Remove 96-well plate containing the cells from the incubator.
26. *Carefully* remove the liquid from each well using a multi-channel pipette. Again, avoid touching the cells with the pipette tips but ensure that all media is removed; tilting the plate will aid this.
27. Pour contents of 15 ml conical tube (containing CellTiter-Fluor™ reagent) into a sterile media trough. Using a multi-channel pipette, *gently* add 100 μl of CellTiter-Fluor™ reagent into each well.
28. Place 96-well plate in the incubator for 60 min.

The Following Steps Do Not Require Sterile Conditions

29. Remove 96-well plate from the incubator, briefly allow to cool to ambient temperature, and then measure fluorescence of individual wells with a microplate reader in fluorescence mode. If using Promega's CTF, employ an excitation wavelength of 380-400 nm and an emission wavelength of 505 nm. Read plate at 10 msec/well using low sensitivity.

30. Pour ONE-Glo™ Assay reagent (prepared in Step #23) into a media trough, and then add 100 µl of this reagent into each well of the plate.
31. Carefully agitate the plate to mix the reagents contained in the wells.
32. After 5 min, read the luminescence of individual wells using a luminometer set for a 5 sec pre-shake with a **5 sec/well** read time. A high gain (sensitivity) setting should be used. Ensure that the luminometer is accumulating RLUs over the 5 sec/well read and not averaging the counts/sec.

DAY 3 CYP3A Metabolism and PXR Activation (DPX2-METAB Kit) (If using Promega's Cell-Titer Fluor, ONE-Glo™ and P450-Glo™ with Luciferin-IPA)

THE FOLLOWING STEPS ARE PERFORMED IN A STERILE HOOD

33. Add 6 µl from Promega's Luciferin-IPA to 6 ml of room temperature Tube D (Dosing Medium) contained in a sterile 15 ml conical tube and pour in media trough.
34. Remove 96-well plate from the incubator after 48 h and place into the hood.
35. Carefully remove the liquid from each well using a multi-channel pipette and discard.
36. Add 50 µl from the media trough containing Luciferin-IPA in dosing medium to each well.
37. Return plate to incubator for 60 min at 37°C.
38. During incubation with Luciferin-IPA, prepare a **white** non-sterile 96-well plate that replicates the format of the original plate. Also, pour the contents of Promega's P450-Glo™ Buffer into Luciferin Detection Reagent. Mix by inversion. Transfer into media trough.
39. After 60 minute incubation, remove 96-well plate from the incubator. Carefully transfer 50 µl from each well of the original plate to the corresponding well of the replicate plate. After addition, remove non-sterile plate from hood.
40. Returning to the hood, transfer 10 ml from Tube E to a sterile 15 ml conical tube, and then add 5 µl from Promega's CellTiter-Fluor™. Mix by inversion. Transfer into media trough.
41. Using a multi-channel pipette, gently add 100 µl of CellTiter-Fluor™ reagent into each well of the original 96-well plate containing the cells. Return plate to the incubator for 60 min.

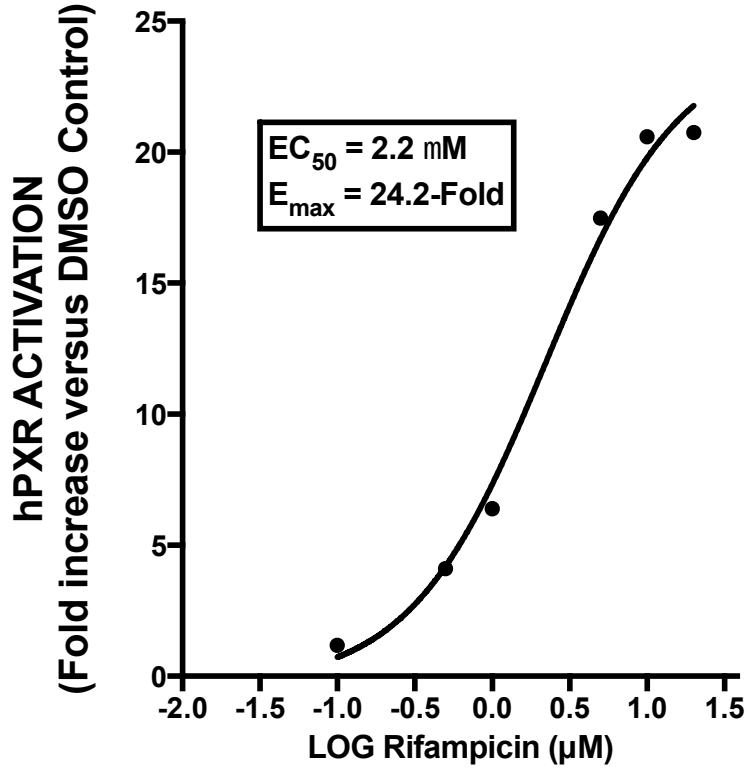
The Following Steps Do Not Require Sterile Conditions

42. Continuing with the replicate plate outside the hood, add 50 µl of P450-Glo™ Buffer/Luciferin Detection Reagent (prepared in step #38) to each well of the replicate plate, and incubate at room temperature for 20 min.
43. After the 20 min incubation with Luciferin Detection Reagent, measure the luminescence of the white 96-well replicate plate with the luminometer set for a 1-5 sec/well read time. A fairly high gain setting should be used. Record results.
44. Add the contents of ONE-Glo™ Assay Buffer to ONE-Glo™ Assay Substrate, cap, and mix by inversion.

45. Following the 60 min incubation with CellTiter-Fluor, remove the original 96-well plate from the incubator, allow to cool to room temperature, and then measure fluorescence of individual wells with a microplate reader in fluorescence mode using an excitation wavelength of 380-400 nm and a emission wavelength of 505 nm.
46. Pour ONE-Glo™ Assay reagent (prepared in Step #44) into a media trough, and add 100 μl of the reagent into each well of the plate.
47. Carefully agitate the plate to mix the reagents contained in the wells.
48. After 5 min, read the luminescence of the individual wells with the luminometer set for a 5 sec pre-shake with a **5 sec/well** read time. A relatively high gain setting should be used. Ensure that the luminometer is accumulating RLUs over the entire 5 sec/well read and not averaging the counts/sec.

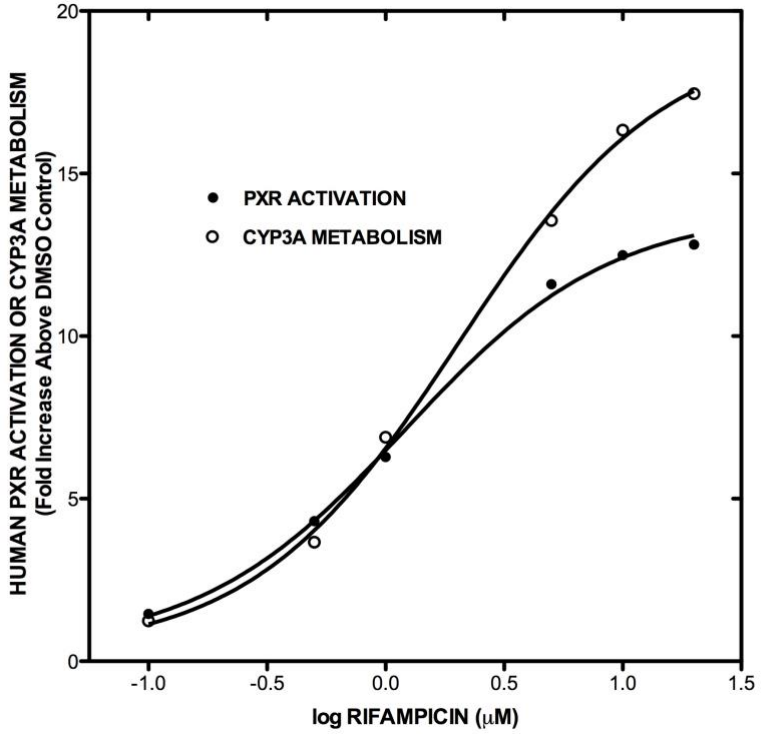
QUANTITATION OF PXR RECEPTOR ACTIVATION

1. Determine the average Relative Luminescence Units (RLU) of the three replicates for each test compound at each dosage. In addition, determine the average Relative Fluorescence Units (RFU) of the three replicates for each test compound at each dosage. RFU values should range between 5000-9000. If the values exceed 9000 RFU, adjust sensitivity or read time/well of the fluorimeter.
2. Determine the mean RLU and RFU for the 0.1% DMSO vehicle control replicates. RLU values for DMSO or media only should range between 100-1000. If values are less than 100, increase sensitivity of the luminescence readout and ensure that the luminometer is accumulating counts over the 5 sec/well read and providing a readout for the 5 sec, not as an average expressed as 1 sec/well.
3. Normalize the luciferase activity for cell viability by dividing the average RLU by the average RFU for each test compound at each dosage as well as for the vehicle control.
4. PXR receptor activation at the individual test compound doses is calculated by dividing the normalized luciferase activity (RLU/RFU) for the test compound doses by that of the normalized DMSO vehicle control. The final data is expressed as fold activation relative to the vehicle control. The use of 5 or more doses of test compound and positive control allows for the derivation of EC_{50} and E_{MAX} values from nonlinear regression analysis of the log dose-response curves. Graphpad Prism (Graphpad Software, La Jolla, CA) and SigmaPlot (Systat Software, San Jose, CA) are among the software programs with built-in equations for deriving these PXR activation kinetic parameters.
5. Test compound values should be compared to those obtained for the positive (rifampicin) control. An example of a typical PXR activation dose-response curve generated with 6 concentrations of rifampicin is shown below.



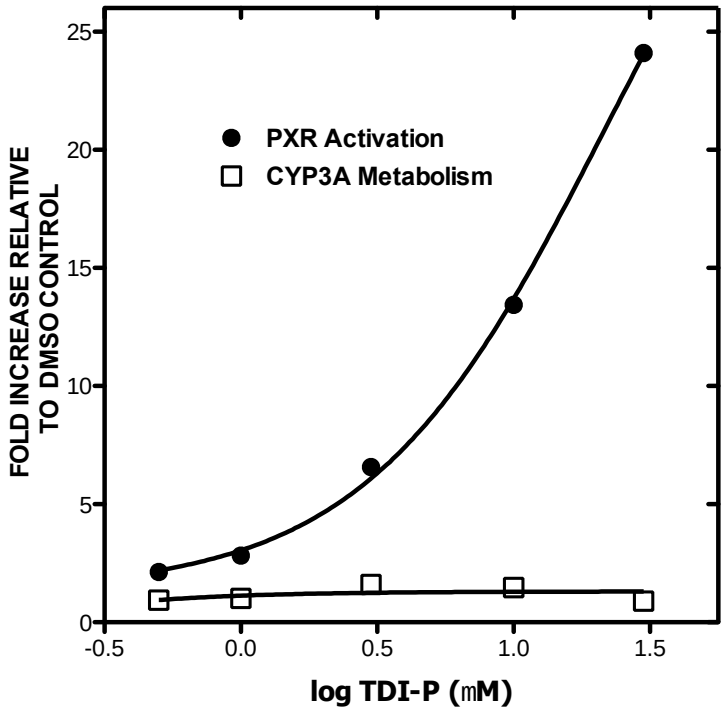
Quantitation of P450 Substrate Metabolism

1. Receptor activation by test compounds, positive controls, and negative (vehicle) controls are determined as described above.
2. To assess whether the test compounds also increased P450 metabolic activity, determine the average RLU of the three Luciferin-IPA replicates for each test compound at each dosage in the white 96-well plates. In addition, determine the average RLU of the three replicates for the DMSO (vehicle) controls.
3. Normalize the P450-Glo™ activity for cell viability by dividing the average RLU by the average viability (RFU) for each test compound at each dosage as well as for the vehicle control.
4. Fold induction by the individual doses of test compound is calculated by dividing the normalized P450-Glo™ luciferase activity (RLU/RFU) for the test compound doses by that of the normalized DMSO vehicle control. The results should be expressed as fold increase (induction) above DMSO-treated cells.



A typical dose-response curve generated in DPX2™ cells treated with 6 concentrations of rifampicin. Both PXR activation and P450 3A metabolism of Luciferin-IPA were monitored in a multiplex fashion. As expected, both metabolic activity and PXR activation were enhanced by rifampicin.

NOTE: *It is not uncommon to obtain extensive PXR activation but negligible induction of P450 substrate metabolism. This happens in cases where the PXR activating compound inhibits the P450 enzyme that metabolizes the P450 Glo™ substrate. The capacity to multiplex Puracyp's Human PXR Activation Assay System allows one to identify such molecules (see on p. 12) as well as those that exhibit cellular toxicity.*



Dose-response curve generated in DPX2™ cells treated with the metabolic inhibitor TDI-P. Both PXR activation and CYP3A metabolism of Luciferin-IPA were monitored in a multiplex fashion. The lack of an increase in Luciferin-IPA metabolism with increasing doses of TDI-P suggests that this compound is indeed a metabolic or time-dependent inhibitor of CYP3A.

TROUBLESHOOTING GUIDE

SYMPTOM	POSSIBLE CAUSE	RESOLUTION
High Well-to-Well Variation	Cells Not Evenly Dispersed Among Wells	**Mix cells thoroughly with medium before seeding.** To facilitate even plating, ensure the cells in Tube A are pipetted up/down at least twice prior to transfer to culture medium. Upon transfer, mix cells with culture medium by pipetting up/down 10 times.
Weak Activation with Potent Positive Control	Inadequate cell number	**Ensure that there are 4 or 6 million viable cells per vial (depending on kit purchased).** Each well of a 96 well plate should receive 40,000 or 60,000 cells/well. To ensure that the cells arrived in good condition following shipping, it is recommended that the cells be counted and viability checked prior to seeding in the 96 well plate.
	Luminometer Settings	**The luminometer used should be one that can be set to accumulate counts over a 5 second/well read.** If the luminometer averages the counts per second, RLU values will be extremely low causing high well-to-well variability and low induction. Ensure that your instrument is set to 5 sec/well read time and is set on the highest sensitivity (gain).
Weak Activation with Test Compounds	Poor Test Compound Solubility	**Test compounds dissolved in DMSO and other solvents precipitate out of solution when added to culture medium**. This often occurs at higher concentrations. Weak activation stemming from poor solubility would be indicated by: a) a peak activation response occurring midway in the dose-response curve, followed by decreasing responses at higher concentrations and; b) no change in cell viability throughout the entire concentration curve.

	Effects on Cell Viability	**Test compound cytotoxicity** can result in weak PXR activation. Cell viability should be assessed using CellTiter-Fluor™ reagent, and the fold PXR activation normalized to the viable cell number for a given test compound. A decline in receptor activation **and** cell viability over the concentration range examined would indicate that the test compound is toxic to the DPX2™cells.
False Positives	Borderline Activation by Non-PXR Ligands	The low levels of PXR activation observed with certain compounds may not be indicative of "false positives". Cut-off values, commonly expressed as % of RIF, should be established to differentiate negative, weak, moderate and potent PXR activators. For example, compounds giving activation < 15% of that observed with 10 μM RIF would be considered negative. Agents giving 15 - 40% of the response observed with 10 μM RIF would be considered weak activators while those eliciting 41 - 69% of the RIF response would be moderate activators. Only those compounds giving **≥** 70% of the response noted with 10 μM RIF would be considered strong or potent PXR activators.
Potent PXR Activation But Weak Induction of CYP3A Metabolism	Test Compound is an Irreversible (Time-Dependent) CYP3A Inhibitor	Certain agents elicit potent activation of PXR together with marked induction of CYP3A enzyme levels but fail to give a corresponding increase in CYP3A-dependent metabolism. This well-documented phenomenon stems from the irreversible, time-dependent inhibition of CYP3A P450s by such compounds during their metabolism by the same enzyme(s).

For any additional questions and/or problems with Puracyp's *Human PXR Activation Assay System*, please contact us by phone at 760-929-9508 or by email at sales@puracyp.com.

V. LIMITED USE DISCLOSURES

Products commercialized by PURACYP, Inc. are intended for RESEARCH PURPOSES ONLY, and not for diagnostic or therapeutic use in humans. Deviations from the protocol(s) described herein will void product warranties, either expressed or implied. In addition, Puracyp will not indemnify utilization of the cells contained in this assay kit.

BY USE OF THIS PRODUCT, RESEARCHER AGREES TO BE BOUND BY THE TERMS OF THIS LIMITED USE STATEMENT. If the researcher is not willing to accept the conditions of this limited use statement, and the product is unused, Puracyp will accept return of the unused product and provide the researcher with a refund. Researchers may use this product for research use only, no commercial use is allowed. Commercial Use means any and all uses of this product and derivatives by a party for monetary or other consideration and may include but is not limited to use in: (1) product manufacture; and (2) to provide a service, information or data; and/or resale of the product or its derivatives, whether or not such product or derivatives are resold for use in research. Researchers shall have no right to modify or otherwise create variations of the cells or the nucleotide sequence of the luciferase gene. No other use or transfer of this product or derivatives is authorized without the prior express written consent of Promega and Puracyp. It is suggested that researchers either: (1) use luminescent assay reagents purchased from Promega Corporation for all determinations of luminescence activity of this product and its derivatives; or (2) contact Promega and Puracyp to obtain a license for use of the product and its derivatives. Researchers may not transfer derivatives to others for research use. With respect to any uses outside this label license, including any diagnostic, therapeutic or prophylactic uses, please contact Puracyp for supply and licensing information. PURACYP DOES NOT MAKE ANY REPRESENTATIONS OR WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED, INCLUDING FOR MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE WITH REGARDS TO THE PRODUCT. The terms of this agreement shall be governed under the laws of the States of California and Wisconsin, USA.

DPX2™ cells are covered by U.S. Pat. No. 5,670,356, European Pat. No. 1341808 and other patents pending owned by Promega Corporation and licensed by Puracyp.

DPX2™ cells are covered by U.S. PATENT NO. 6,756,491 and European Pat. No. 1051479 owned by the Salk Institute and licensed by Puracyp.

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.

DISCLAIMER

PURACYP, INC. MAKES NO CLAIMS OR WARRANTIES, EITHER IMPLIED OR EXPRESSED, AS TO THE PHYSIOLOGICAL EFFECT OF ANY COMPOUND IN VIVO ASSAYED WITH OUR TECHNOLOGY. PURACYP DOES NOT WARRANT THAT SIMILAR EFFECTS WILL OCCUR IN WHOLE ORGANISMS (*IN VIVO*).

COMPOUNDS THAT ARE TOXIC OR INHIBIT CELL CYCLE EVENTS MAY NOT EXHIBIT INDUCTION IN PURACYP'S CELL LINES BUT RECEPTOR ACTIVATION MAY BE OBSERVED IF A VIABILITY ASSAY IS PERFORMED AND RESULTS NORMALIZED TO THAT ASSAY.

IF THE ABOVE TERMS ARE NOT ACCEPTABLE, PROMPTLY CONTACT PURACYP. FAILURE TO PROMPTLY CONTACT PURACYP IN WRITING WITHIN TWO DAYS OF DELIVERY CONSTITUTES ACCEPTANCE OF THE ABOVE TERMS. DEVIATIONS FROM THE ABOVE PROTOCOLS WILL VOID WARRANTIES.

VI. REFERENCES

Raucy JL, Warfe L, Yueh M-F, Allen SW: A cell-based reporter gene assay for determining induction of CYP3A4 in a high volume system. *J Pharmacol Exp Ther* 303: 412-423, 2002.

Yueh M-F, Kawahara M, Raucy JL: High-volume bioassays to assess CYP3A4-mediated drug interactions: Induction and inhibition in a single cell line. *Drug Metab Disp* 33:38-48, 2005

Yueh M-F, Kawahara M, Raucy JL: Cell-based high-throughput bioassays to assess induction and inhibition of CYP1A enzymes. *Toxicology In Vitro*. 19:275-287, 2005.

Trubetskoy O, Marks B, Zielinski T, Yueh M-F, Raucy JL: A simultaneous assessment of CYP3A4 metabolism and induction in the DPX-2 cell line. *AAPS Journal* 07(01): E6-E13, 2005.

Sinz MW, Devin Pray, Raucy JL: The utility of stable cell lines to assess species differences in PXR transactivation. *Drug Metab Lett* 1: 147-152, 2007.

Lipp E: Tackling Drug-interaction issues early on. *Genetic Engineering & Biotechnology News*, June 15: 14-20, 2008.

Raucy JL, Lasker JM: Current *in vitro* high throughput screening approaches to assess nuclear receptor activation. *Curr Drug Metab* 11:806-814, 2010.

Shukla, SJ, Sakamuru, S, Huang, R, Moeller, TA, Shinn, P, VanLeer, D, Auld, DS, Austin, CP and Menghang, X. Identification of clinically used drugs that activate pregnane X receptors. *Drug Metab Disp* 39: 151-159, 2011.

Fahmi, O., Raucy, J. L., Ponce, E. and Lasker, J. M. The Utility of DPX Cells for predicting CYP3A4 induction-mediated drug-drug interactions and associated structure activity relationships. *Drug Metab. Disp*.40: 2204-2211, 2012

Raucy, J.L. and Lasker, J. M. Cell-based systems to assess nuclear receptor activation and their use in drug development. *Drug Metab. Rev.* 45:101-109, 2013.

Raucy, J. L. Human Pregnane X Receptor (hPXR) Activation Assay in Stable Cell Lines. In Optimization in Drug Discovery: In Vitro Methods, Methods in Pharmacology and Toxicology. Ed. Caldwell, G. W. and Yan Zhengyin, pp.171-193, Springer Science, NY. 2014.

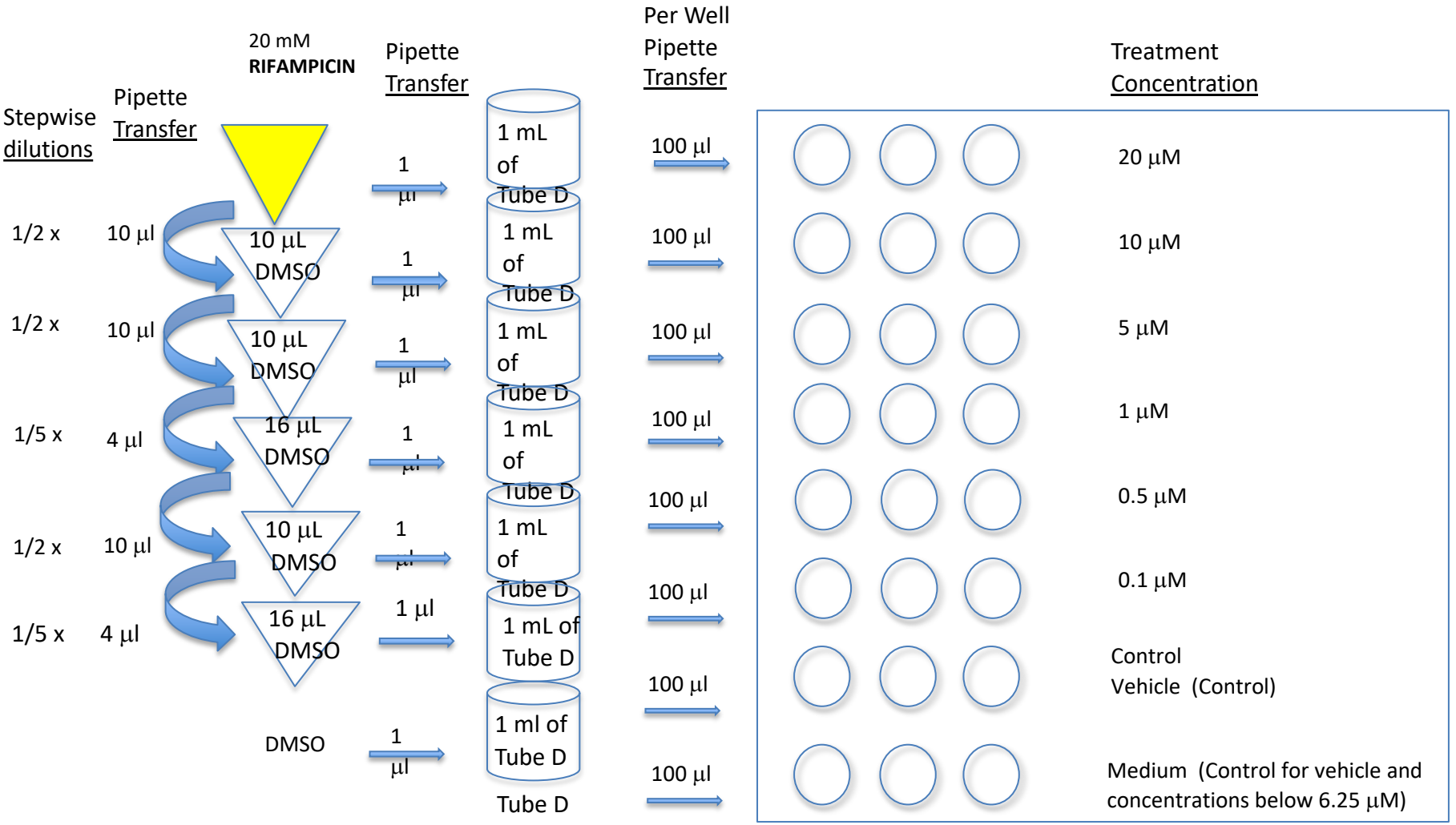
Pinne, M. and Raucy, J. L. Advantages of cell-based high-volume screening assays to assess nuclear receptor activation during drug discovery. *Expert. Opin.Drug Disc*. 9:669-686, 2014.

Pinne, M., E. Ponce and J. L. Raucy. Transactivation Assays that Identify Indirect and Direct Activators of Human Pregnane X Receptor (PXR, NR1I2) and Constitutive Androstane Receptor (CAR, NR1I3). Drug Metabolism Letters. Vol: **11**: No: 2. 2017.

Pinne, M. and Raucy, J. L. Cytochrome P450 Gene Regulation: Reporter Assays to Assess Pregnane X Receptor (PXR, NR1I2) Activation. In Cytochrome P45: In Vitro Methods & Protocols. Methods in Phramacology & Toxicology.Ed. Caldwell, G. W. and Yan Zhengyin, pp.171-193, Springer Protocols. Humana Press, NY. 2021.

APPENDIX 1 FOR DPX2-ACTIV & DPX2-METAB

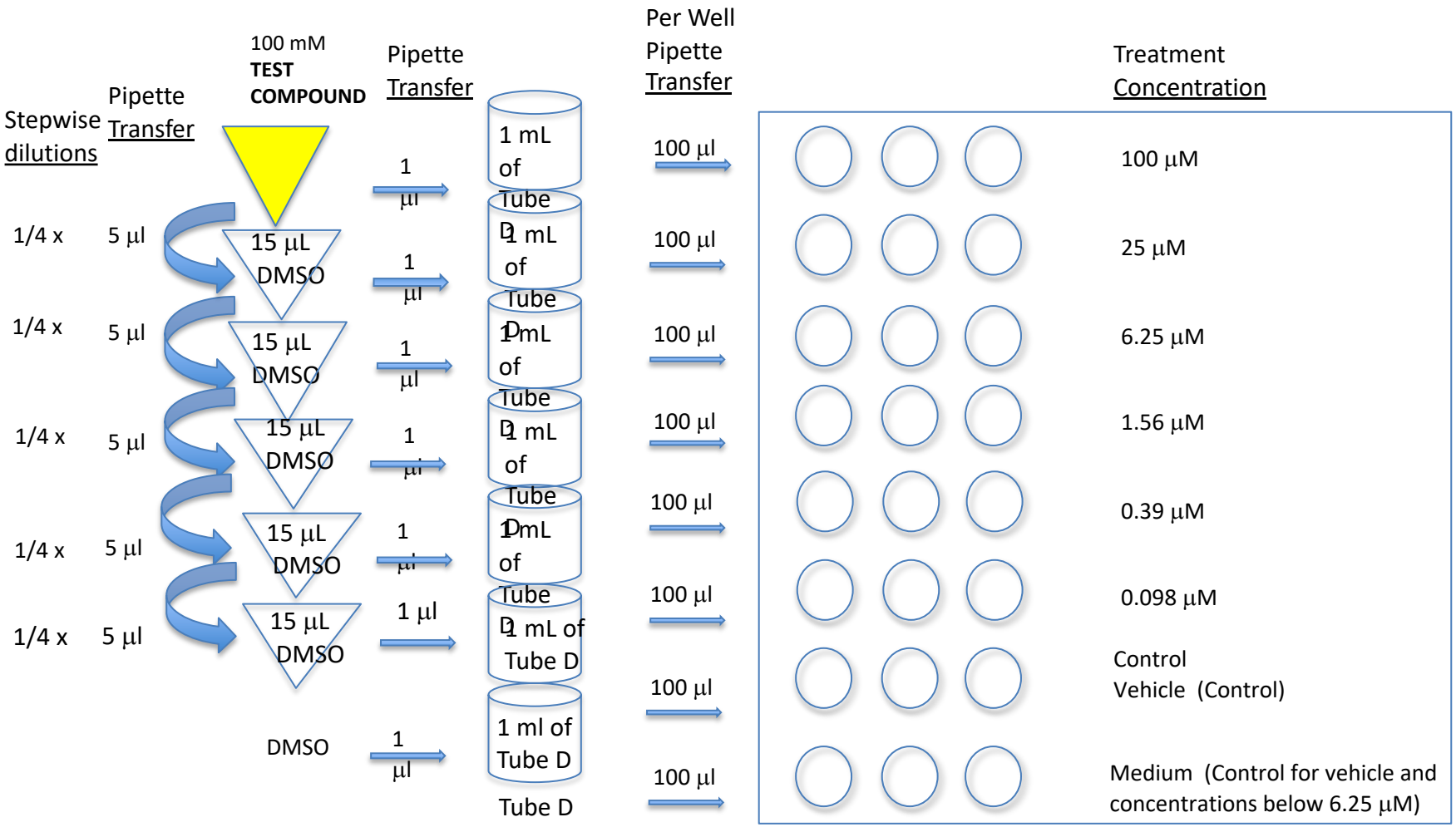
Dilution Scheme for ***Dosing Positive Control***



All test compound dilutions are made with neat DMSO, after which the individual solutions are added to dosing media at a ratio of 1:1000.

APPENDIX 2 FOR DPX2-ACTIV & DPX2-METAB

Dilution Scheme for ***Dosing*** Test Compounds



All test compound dilutions are made with neat DMSO, after which the individual solutions are added to dosing media at a ratio of 1:1000.