HUMAN ARYL HYDROCARBON RECEPTOR (AhR) ACTIVATION ASSAY SYSTEM

Technical Manual for 96-Well Format Assay
Catalog# 1A2-DRE-96-001 and 1A2-DRE 96-002

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HUMAN AhR NUCLEAR RECEPTOR ACTIVATION ASSAY, WITH ASSESSMENT OF CELL VIABILITY AND CYP1A2 SUBSTRATE METABOLISM IN A 96-WELL FORMAT

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 ; Email: sales@puracyp.com

I. DESCRIPTION

Puracyp's nuclear receptor activation kits are all-inclusive cell-based assay systems. The kits incorporate mycoplasm-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 96-well assay. The kits also include a cell culture-ready assay plate, optimized cell culture medium, cell dosing medium, a positive control specific for the nuclear receptor, CellTiter-Fluor™ for determining cell viability, luciferase detection reagents and, if so ordered, a P450-Glo™ substrate for assessing induction of CYP450 enzyme activity. The primary application of these assay kits is to identify agents with the ability to activate PXR and/or AhR, thereby inducing drug-metabolizing enzymes and ABC transporters.

Puracyp's *Human AhR Activation Assay System* utilizes 1A2-DRE™ cells constructed with a proprietary process for producing stably-transfected cell lines. 1A2-DRE™ cells harbor the human AhR gene and a luciferase reporter gene linked to the human CYP1A2 promoter and 3 copies of the dioxin response element (DRE) enhancer. The 1A2-DRE™ 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 1A2-DRE™ cells and other stable cell lines is given in this manual.

Puracyp's *Human AhR Activation Assay System* also includes CellTiter-Fluor™, a cell viability detection reagent, and ONE-Glo™, a luciferase detection reagent, from Promega Corporation. Since the luciferase reporter gene is stably integrated into Puracyp's 1A2-DRE™ cells, the assay gives extremely low background, high sensitivity, and a broad linear dynamic range typical of bio-luminescence reporter gene technology. Further details on the use of CellTiter-Fluor™, ONE-Glo™ and the P450-Glo™ CYP1A2 Assay with Luciferin-1A2 can be found at www.promega.com.

In a typical assay, 1A2-DRE™ cells are first dispensed into the wells of the assay plate, and then placed in a CO_2 incubator at 37^{o}C to equilibrate overnight. The following day, the cells are treated with user's test compounds and the appropriate positive control. Since the 1A2-DRE™ 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^{o}C for 24 h. The dosing medium is then discarded, cell viability is assessed fluorimetrically with CellTiter-Fluor™, and luciferase activity then measured using ONE-

Glo™. The luminescence light intensity is directly proportional to the extent of AhR activation and accompanying gene transcription in the 1A2-DRE™ cells.

II. ASSAY KIT COMPONENTS & STORAGE CONDITIONS

Puracyp's *Human AhR Activation Assay System* (Product 1A2-DRE™-96-001 and 1A2-DRE™-96-002) 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 1A2-DRE™ cells, media and detection reagents are intended for a single-use. Once 1A2-DRE™ cells have been thawed, they can neither be refrozen nor cultured for future use due to the utilization of a cell-arrest agent during cellular preparation. Therefore, if an entire 96-well plate is not seeded, the remaining cells should be discarded

Assay kits are shipped on dry ice and the 1A2-DRE cells are shipped in a liquid nitrogen dry shipper. **If the 1A2-DRE™ 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 90 days. Individual expiration dates are provided with each kit.

KIT COMPONENT	***AMOUNT***	***STORAGE TEMPERATURE***
Tube **A** – 1A2-DRE™ Cells	6 million x 1 vial	Liquid Nitrogen
Tube **B** - Positive Control (20 mM)	1 x 10 µl	-20° C
Tube **C** - Culture Media	1 x 10 mL	-20° C
Tube **D** - Dosing Media	1 x 35 mL	-20° C
Tube **E**	1 x 10 mL	-20° C
Tube **F** - ONE-Glo™ Assay Buffer	1 x 10 mL	-20° C
Tube **G** - ONE-Glo™ Assay Substrate, Lyophylized	1 vial	-20° C
Tube **H** - P450-Glo™ Buffer	1x 10 ml	-20° C
Tube **I** - Luciferin Detection Reagent, Lyophylized	1 vial	-20° C
Tube **J** - CellTiter-Fluor™	1 x 5 µl	-20° C
Tube **K** - Luciferin-1A2 + D-Cysteine (100 µl)	1 + 1 vial	-20° C
96-Well Microtiter Plate	1	Ambient
D-CYSTEINE	100 µl	-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^{O} C water bath
- Sterile multi-channel media basins or reservoirs
- 70% ethanol mixture
- Multi-channel (8- or 12-channel) pipette & sterile tips

FOR DAY 1

- Cell culture-rated hood
- Humidified 5% CO_2 /37°C incubator
- 37^{O}C water bath
- Sterile 1.5 ml tubes for making DMSO dilutions of test compounds
- Sterile 96 deep well plate (2 ml/well capacity, Eppendorf cat.# 9400002001) to make test compound doses
- Sterile media trough or basin
- DMSO or other solvent for use as a test compound diluent and as the negative control
- Multi-channel (8 or 12) pipette & sterile tips

FOR DAY 2 (Receptor Activation, 1A2-DRE-96-001 kit)

- 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.
- 2 x Sterile Media troughs
- 15 ml sterile conical centrifuge tube
- Multi-channel pipette & sterile tips

***FOR DAY 3* (P450 Metabolism, 1A2-DRE-96-002 kit)**

- **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.
- 3 x Sterile Media trays
- Multi-channel pipette & sterile tips
- 2 x 15 ml sterile conical centrifuge tubes
- Sterile Phosphate buffered saline (PBS) at room temperature

IV. ASSAY PROCEDURE

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

Puracyp's *Human AhR Activation Assay* incorporates one overnight and a single 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 - 3.5 h** to complete, depending on the number of dilutions and compounds to be tested. *Steps 22-49* are performed on Day 2, and require **1.5-5 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 96-well plate from kit 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^{O} C water bath until media has thawed and warmed to 37^{O}C, and transfer 10 ml into a sterile trough.
6. Place Tube A containing the 1A2-DRE™ cells into a 37^{O}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 medium from Tube C.
8. Remove 2 ml of the cell-media mixture from trough, and use it to rinse out Tube A. Transfer contents back to the media trough.

9. Uniformly mix cells with media by pipetting up and down at least 10X with a 10 ml pipette. It is recommended that the cells be counted to ensure there are 6 million viable cells/vial.
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 the seeding of each row/column.
11. Place seeded plate into humidified 5% CO_2 /37°C incubator overnight.

DAY 1

12. Place Tube B from kit at room temperature.
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 (Tube B) dilutions as described in **Appendix 1.**
16. Prepare Test Compound dilutions as 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 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 media containing test agent, positive control or vehicle control to the plate wells. *Media 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** (1A2-DRE-96-001) OR **48 h** (1A2-DRE-96-002)[A].

NOTE: A 48 H INCUBATION OF CELLS IS EMPLOYED WHEN P450-GLO™ METABOLISM WILL BE ASSESSED.

[A]Compounds other than BNF 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 Receptor Activation (1A2-DRE-96-001 kit)

22. Allow Tubes E, F, G & J to reach room temperature.
23. Add the contents of Tube F (ONE-Glo™ Assay Buffer) to Tube G (ONE-Glo™ Assay Substrate), cap, and invert the bottle 3X to mix.
24. Transfer 10 ml from Tube E to a sterile 15 ml conical tube, and add 5 µl from Tube J (CellTiter-Fluor™). Mix by inverting tube. Pour into sterile media tray.
25. Remove 96-well plate 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 tray. 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 using an excitation wavelength of 380-400 nm and an emission wavelength of 505 nm. Read plate at 10 msec/well.
30. Pour ONE-Glo™ Assay reagent (prepared in Step #23) into a media tray, 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 the counts over the 5 sec/well read time and not averaging the RLUs/sec.

DAY 3 P450 Metabolism and Receptor Activation (1A2-DRE-96-002 kit)

THE FOLLOWING STEPS ARE PERFORMED IN A STERILE HOOD

33. Allow Tubes E, F, G, H, I, J, K and D-cysteine to reach room temperature.
34. Add 6 µl from Tube K (Luciferin-1A2) to 6 ml of room temperature sterile phosphate buffered saline (PBS) to a sterile 15 ml conical tube, mix and pour into media tray.
35. Remove 96-well plate from the incubator after 24 h and place into the hood.
36. Carefully remove the liquid from each well using a multi-channel pipette and discard. Wash cells with 100 µl/well of phosphate buffered saline (PBS). Gently discard PBS.
37. Add 50 µl from the media tray containing Luciferin-1A2 in PBS to each well.

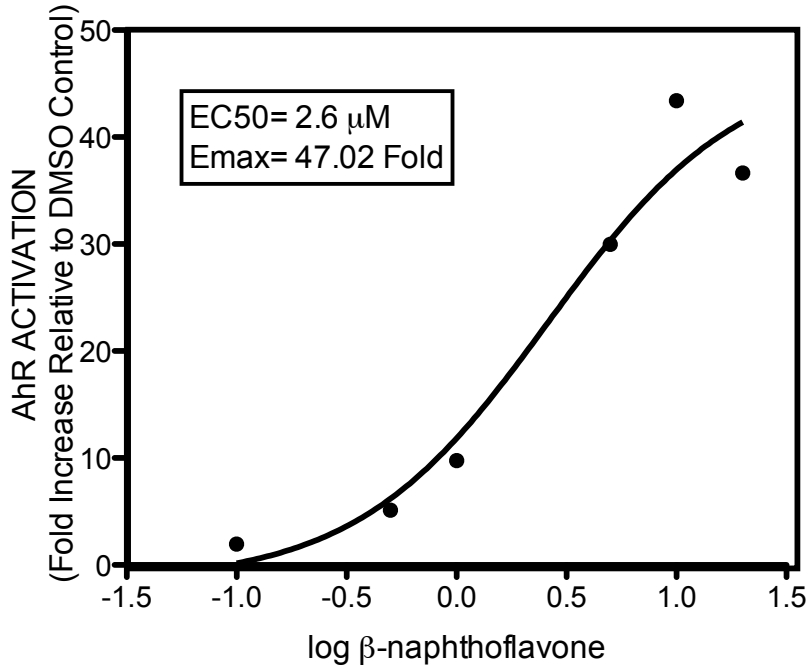
38. Return plate to incubator for 180 min (3 h) at 37°C.
39. During incubation with Luciferin-1A2, pour the contents of Tube H (P450-Glo™ Buffer) into Tube I (Luciferin Detection Reagent). Mix by inversion and add 100 µl of D-cysteine. Mix by inversion and pour into media trough. Also, prepare a replicate white non-sterile 96 well plate that replicates the format of the original plate.
40. Following the 3 h 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. Remove non-sterile plate from hood.
41. Following transfer of Luciferin-1A2 substrate to replicate plate and removal from hood, back in the hood transfer 10 ml from Tube E to a sterile 15 ml conical tube, and then add 5 µl from Tube J (CellTiter-Fluor™). Mix by inversion. Pour into media trough.
42. 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

43. Continuing with the replicate plate out side the hood, add 50 µl of P450-Glo™ Buffer/Luciferin Detection Reagent containing the D- cysteine (prepared in step # 39) to each well of the replicate plate, and incubate at room temperature for 20 min.
44. After the 20 min incubation with Luciferin Detection Reagent, measure the luminescence of the white non-sterile 96-well plate with the luminometer set for a 1-5 sec/well read time. A fairly high gain (sensitivity) setting should be used. Record data.
45. Add the contents of Tube F (ONE-Glo™ Assay Buffer) to Tube G (ONE-Glo™ Assay Substrate), cap, and mix by inversion.
46. 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 an emission wavelength of 505 nm.
47. Pour ONE-Glo™Assay reagent (prepared in Step #45) into a media trough, and add 100 µl of the reagent into each well of the plate.
48. Carefully agitate the plate to mix the reagents contained in the wells.
49. 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. Ensure that the luminometer is accumulating RLUs over the entire 5 sec/well read and not averaging the counts/sec. A relatively high gain setting should be used.

QUANTITATION OF AhR 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 5000-11,000. If the values exceed 11,000 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 should range 1000-6000. If values are less than 1000, increase the sensitivity of the luminescence readout and ensure that the luminometer is accumulating counts over the 5 sec/well read and providing a readout per 5 sec, not as an average expressed as 1 sec/well.
3. Normalize the luciferase activity for cell viabililty by dividing the average RLU by the average RFU for each test compound at each dosage as well as for the vehicle control.
4. AhR 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 AhR activation kinetic parameters.
5. Test compound values should be compared to those obtained for the positive (BNF) control. An example of a typical AhR activation dose-response curve generated with 6 concentrations of BNF is shown below.

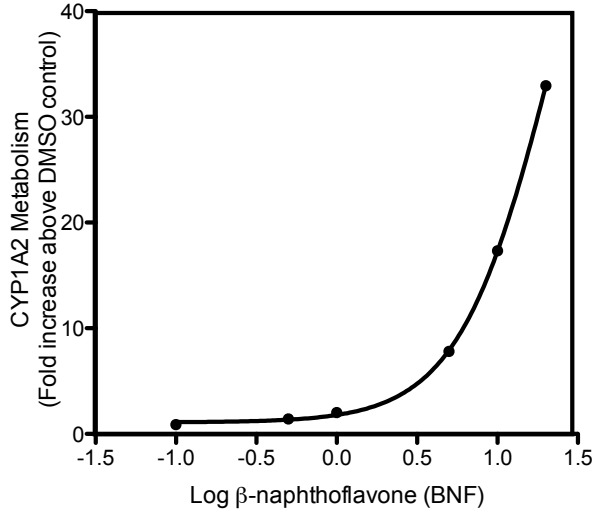


A typical dose-response curve for AhR activation generated in 1A2-DRE™ cells treated with 6 concentrations of BNF.

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-1A2 replicates for each test compound at each dosage in the replicate 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 viabililty (determined on the original plate) 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™ (Luciferin –1A2) 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 dose-response curve for CYP1A2 metabolism generated in 1A2-DRE™ cells treated with 6 concentrations of BNF. P450 1A2 metabolism of Luciferin-1A2 was monitored in a multiplex fashion. As expected, metabolic activity was enhanced by BNF.

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

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 6 million viable cells per vial.** Each well of a 96 well plate should receive 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) possible.

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 media**. 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 AhR activation. Cell viability should be assessed using CellTiter-Fluor™ reagent, and the fold AhR 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 1A2-DRE™cells.
False Positives	Borderline Activation by Non-AHR Ligands	The low levels of AhR activation observed with certain compounds may not be indicative of "false positives". Cut-off values, commonly expressed as % of BNF, should be established to differentiate negative, weak, moderate and potent AhR activators. For example, compounds giving activation < 15% of that observed with 10 μM BNF would be considered negative. Agents giving 15 - 40% of the response observed with 10 μM BNF would be considered weak activators while those eliciting 41 - 69% of the BNF response would be moderate activators. Only those compounds giving ≥ 70% of the response noted with 10 μM BNF would be considered strong or potent AhR activators.

Potent AHR Activation But Weak Induction of CYP1A2 Metabolism	Test Compound is an Irreversible (Time-Dependent) CYP1A2 Inhibitor	Certain agents elicit potent activation of AhR together with marked induction of CYP1A2 enzyme levels but fail to give a corresponding increase in CYP1A2-dependent metabolism. This well-documented phenomenon stems from the irreversible, time-dependent inhibition of CYP1A2 by such compounds during their metabolism by the same enzyme.

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

V. LIMITED USE DISCLOSURES

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1A2-DRE™ 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.

ONE-Glo™ and CellTiter-Fluor™ are subject to patents pending by Promega Corporation.

P450-Glo™ CYP1A2 Assay with Luciferin-1A2 is covered by U.S. Pat. Nos. 6,602,677 and 7,724,584, European Pat. No. 1131441 and other patents pending owned by Promega Corporation.

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.

Additional information on ONE-Glo™, CellTiter-Fluor™ and the P450-Glo™ CYP1A2 Assay with Luciferin-1A2 can be found at www.promega.com.

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VI. REFERENCES

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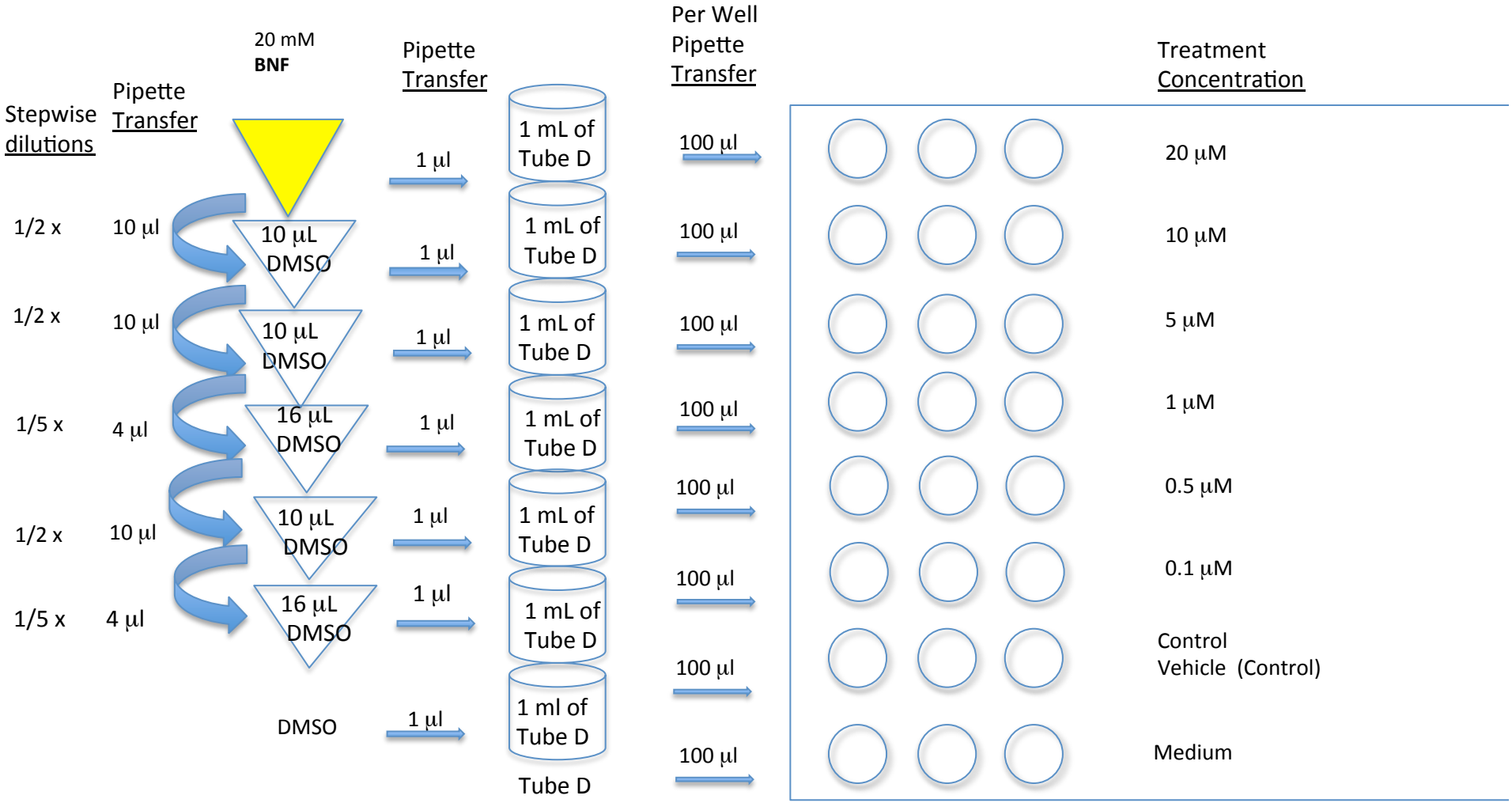
APPENDIX 1 FOR DRE-1A2-96-001 AND DRE-1A2-96-002

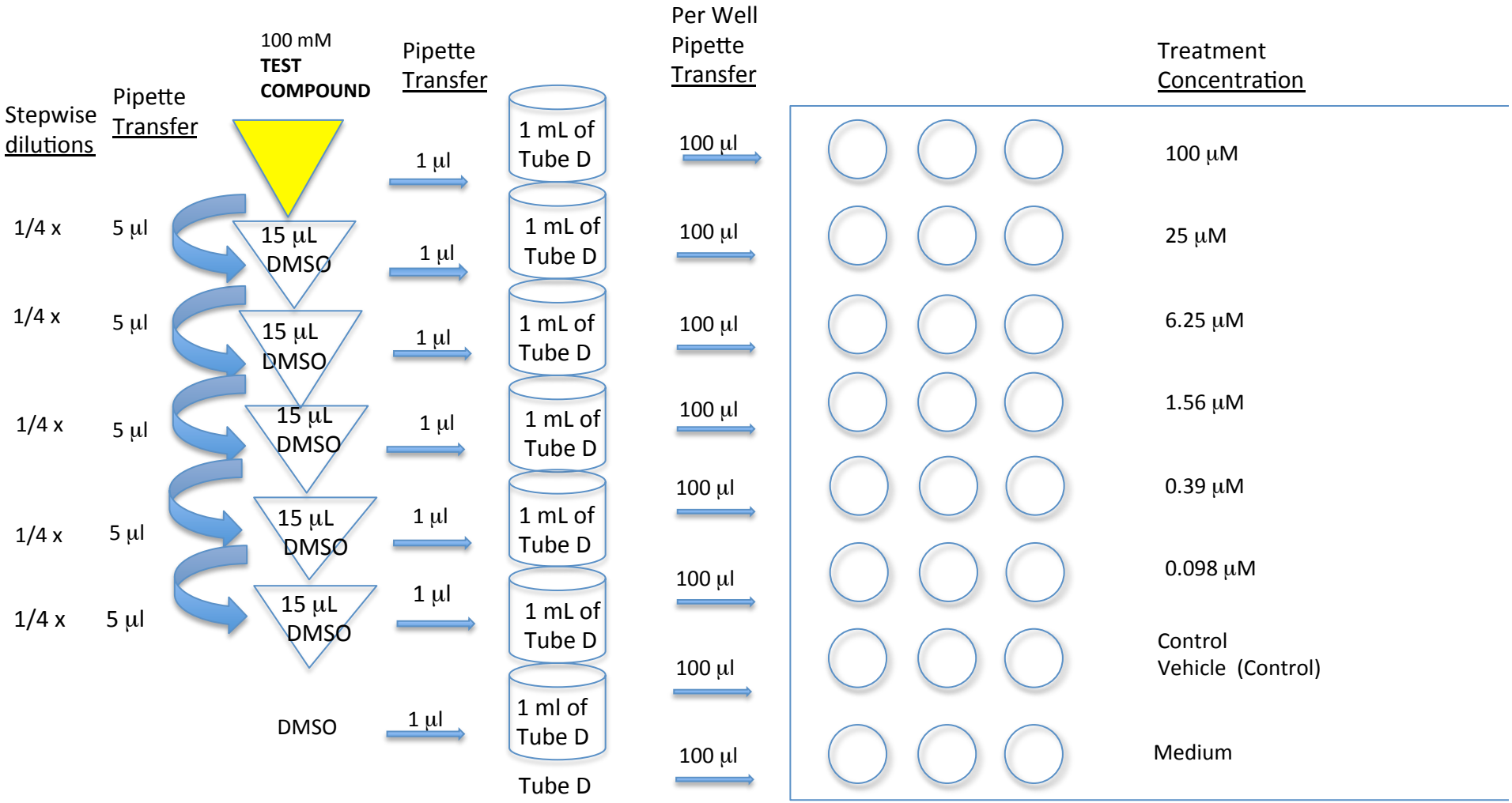
Dilution Scheme for ***Dosing Positive Control***



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

APPENDIX 2 FOR DRE-1A2-96-001 AND DRE-1A2-96-002

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.

APPENDIX 3: QUICK REFERENCE CARD

HUMAN AhR NUCLEAR RECEPTOR ACTIVATION ASSAY WITH ASSESSMENT OF CELL VIABILITY IN A 96-WELL FORMAT (1A2-DRE-96-001)

USE STERILE TECHNIQUE FOR STEPS 1-17

KIT RECEIPT DAY

1. Thaw Tube C by warming in a 37^{o} C water bath, and transfer 10 ml into a media trough.
2. Place Tube A containing 1A2-DRE cells in a 37^{o} C water bath until cell suspension has thawed.
3. Pipette cells into the media trough containing 10 ml of Reagent C, and mix by pipetting up-and-down 10 times. Count cells and determine viability.
4. Transfer 100 µl of cell mixture into plate wells, keeping cells suspended while pipetting.
5. Place seeded plate into a humidified CO_2 incubator at 37^{o}C overnight.

DAY 1

6. Thaw Tube D in a 37^{o}C water bath.
7. Allow Tube B to thaw at room temperature.
8. Prepare series of Test Compound and Positive Control dilutions in DMSO (see Appendices 1 & 2) followed by 1:1000 dilutions in dosing medium (Tube D).
9. Remove plate from the incubator, and carefully aspirate and discard the media from each well. Do not disturb the cells during the aspiration process.
10. Transfer 100 µl of each test compound concentration into pre-marked wells. Repeat with positive controls and vehicle controls.
11. Return plate to the incubator for a 24[A] h exposure to the treatments.

DAY 2

12. Thaw Tube J containing CellTiter-Fluor™.
13. Thaw Tube E and transfer 10 ml to a sterile 15 ml centrifuge tube. Add 5 µl of CellTiter-Fluor™ (Tube J). Mix by inversion and pour into media trough.
14. Thaw Tube F containing ONE-Glo™ Luciferase Assay Buffer, and add to Tube G containing. ONE-Glo™ Assay Substrate; mix vial several times by inversion. Pour into media trough.
15. Remove plate from the incubator, and carefully aspirate and discard all dosing medium containing test compounds, positive controls, and negative (vehicle) controls.
16. Add 100 µl of Tube E containing the CellTiter-Fluor™ reagent (prepared in Step 13) to each well.
17. Return plate to the incubator for 60 min.
18. After incubation, measure fluorescence of individual wells with a multi-mode microplate reader[B] set for fluorescence using an excitation wavelength of 380-400 nm and an emission wavelength of 505 nm.
19. Remove plate from instrument, and add 100 µl of ONE-Glo™ Assay reagent to each well. Agitate plate to mix, and incubate for 5 min at room temperature.
20. Measure luminescence of individual wells with the multi-mode reader set for a 5 sec pre-shake and a 5 sec/well read. A high instrument gain (sensitivity) setting should be used.
21. Receptor activation is calculated according to the method given in the *Complete Protocol*.

[B]A stand-alone fluorimeter and luminometer can also be used

APPENDIX 4: QUICK REFERENCE CARD

HUMAN AhR NUCLEAR RECEPTOR ACTIVATION ASSAY WITH CELL VIABILITY AND CYP1A2 SUBSTRATE METABOLISM IN A 96-WELL FORMAT (1A2-DRE-96-002)

USE STERILE TECHNIQUE FOR STEPS 1-20

KIT RECEIPT DAY

1. Thaw Tube C by warming in a 37°C water bath, and transfer 10 ml into a media trough.
2. Place Tube A containing 1A2-DRE cells in a 37° C water bath until cell suspension has thawed.
3. Pipette cells into the media trough containing 10 ml of Reagent C, and mix by pipetting up-and-down 10 times. Count cells and determine viability.
4. Transfer 100 µl of cell mixture into plate wells, keeping cells suspended while pipetting.
5. Place seeded plate into a humidified CO_2 incubator at 37°C overnight.

DAY 1

6. Thaw Tube D in a 37°C water bath.
7. Allow Tube B to thaw at room temperature.
8. Prepare series of Test Compound and Positive Control dilutions in DMSO followed by 1:1000 dilutions in Dosing Medium (Tube D) (see Appendices 1 & 2).
9. Remove plate from the incubator, and carefully aspirate and discard the medium from each well. Do not disturb the cells during the aspiration process.
10. Transfer 100 µl of each test compound concentration into pre-marked wells. Repeat with positive controls and vehicle controls.
11. Return plate to incubator for 48 h[A]

DAY 3

12. Thaw Tubes J and K containing CellTiter-Fluor™ and Luciferin-IPA, respectively.
13. Allow Tubes D, E, F, G, H, I, and D-Cysteine to reach room temperature.
14. Add 6 µl of Tube K to 6 ml of room temperature sterile phosphate buffered saline (PBS). Mix by inversion. Pour into media trough.
15. Remove 96-well plate from incubator and carefully remove media from wells and discard. Wash cells with 100 ul/well of sterile phosphate buffered sale (PBS) and aspirate PBS and discard.
16. Add 50 µl of the Luciferin-IPA reagent (prepared in step 14) to each well. Return plate to incubator for 180 min.
17. During the incubation, prepare a non-sterile white 96-well plate that replicates the format of the original plate. Also, pour Tube H (P450-Glo buffer) into Tube I (Luciferin Detection Reagent) and mix by inversion. Add 100 µl of D-cysteine and mix again. Pour into media trough.
18. After the 3 h incubation, remove 96-well plate from the incubator, and transfer 50 µl from each well to the corresponding well of the replicate plate and remove plate from hood.
19. Back in the hood, transfer 10 ml of Tube E to a 15 ml sterile conical tube, add 5 µl of CellTiter-Fluor™ (Tube J), and mix by inversion. Pour into media trough.

20. 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.
21. Add 50 µl of Luciferin Detection Reagent/P450-Glo Buffer containing the D-cysteine (prepared in step # 17) to each well of the replicate non-sterile plate that is outside of the hood, agitate the plate, and incubate at room temperature for 20 min.
22. After 20 min incubation with Luciferin Detection Reagent, determine luminescence of the wells of the non-sterile white 96-well plate with the luminometer set for a 1-5 sec/well read time. A relatively high gain (sensitivity) setting should be used. Record results.
23. Add Tube F containing ONE-Glo™ Luciferase Assay Buffer to Tube G containing ONE-Glo™ Assay Substrate; mix by inversion. Pour into media trough.
24. After 60 min incubation at 37°C (see step #20), remove original 96-well plate from incubator and measure fluorescence of individual wells with multi-mode microplate reader[B] set to the fluorescence mode using an excitation wavelength of 380-400 nm and an emission wavelength of 505 nm.
25. Remove plate from instrument, and add 100 µl of ONE-Glo™ Assay reagent (prepared in step # 23) to each well. Agitate plate to mix, and incubate for 5 min at room temperature.
26. Measure luminescence of individual wells with the multi-mode reader set for a 5 sec pre-shake and a **5 sec/well** read. A high instrument gain (sensitivity) setting should be used.
27. Receptor activation is calculated according to the method given in the *Complete Protocol*.

CellTiterFluor™ and ONE-Glo™ are trademarks of the Promega Corporation.

[A] Compounds other than BNF may be less stable in culture at 37°C, and thus may not function as AhR ligands for the entire treatment period. In those instances, the user may want to perform two 24 h treatments, which will require that Day 1 Steps #8-11 are repeated on Day 2.

[B] A stand-alone fluorimeter and luminometer can also be used.