Introduction

A variety of molecular targeted drug screening assays have been developed by the Developmental Therapeutics Program in collaboration with various investigators. A number of additional assay are currently under development. These vary in format, ranging from simple cell-free assays to those involving cell lines engineered to express particular reporter systems. The screening assays are intended for use in conjunction with chemical libraries to support discovery of novel molecular targeted agents, or drug leads, relevant to treatment of cancer, AIDS-associated malignancies, HIV-1 disease or opportunistic infections. For a recent review of the process, see Shoemaker, et al. Application of High-Throughput, Molecular-Targeted Screening to Anticancer Drug Discovery. Current Topics in Medicinal Chemistry 2:229-246, 2002. A downloadable copy of this review is available at: <http://spheroid.ncifcrf.gov/stb/staff/shoemaker/shoemaker.cfm>.

Data generated during assay development (screening data on the NCI Training Set) and results from high-throughput screening of the NCI Diversity Set are available on this page.

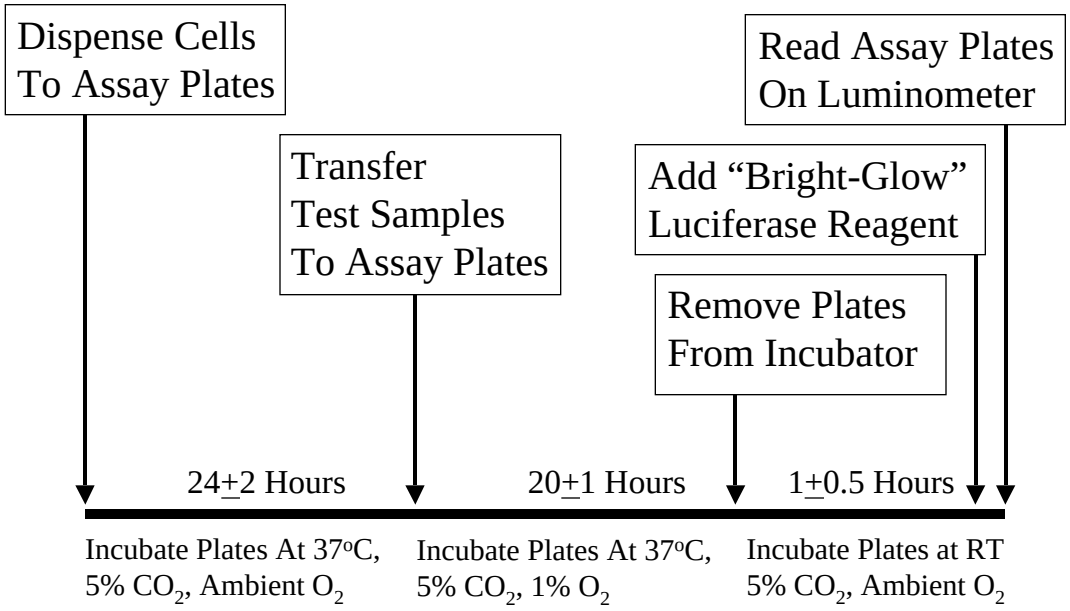
HIF-1 AS A TARGET FOR DEVELOPMENT OF NOVEL CANCER THERAPEUTICS

- HIF-1 IS CRITICAL FOR THE EXPRESSION OF GENES SUCH AS VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF), GLYCOLYTIC ENZYMES AND INDUCIBLE NITRIC OXYDE SYNTHASE (iNOS).
- GENETIC DELETION OF THE HIF-1a SUBUNIT IN MOUSE EMBRYOS RESULTED IN DEVELOPMENTAL ARREST AND EMBRYONIC LETHALITY BY E11.
- CELLS LACKING A FUNCTIONAL HIF-1 SHOW IMPAIRED GROWTH AND REDUCED VASCULARITY IN TUMOR XENOGRAFT MODELS.
- OVEREXPRESSION OF HIF-1a HAS BEEN DEMONSTRATED IN SEVERAL HUMAN

Reference

Identification of novel inhibitors of HIV-1 transcriptional activation and VEGF expression using a high throughput targeted screen. Rapisarda, A, Uranchimeg, B, Scudiero, D.A.,Selby, M.H., Stevenson, T.J., Sausville, E.A., Shoemaker, R.H., Melillo, G *Cancer Researc*h (in press August 2002).

Schematic Protocol for HIF-1 Targeted HTS Assay



A HIF-1 LUMINESCENCE REPORTER ASSAY USING U251 CELL LINE BACKGROUND

I. CELL MAINTENANCE:

A. U251-HIF-regular cell culture protocol is followed. Pull repository vial, thaw, and place in conical tube and pellet. Decant supernatant and resuspend in T-75 flask containing 25 ml media (RPMI Phenol red-free) containing 1% L-Glutamine, 10% FBS and G418 at *500* µg/ml final concentration for selective propagation.

II. SPLIT WEEKLY:

A. U251-HIF- Tissue culture is done twice weekly. 35 ml media are added to T150 tissue culture flasks.
And cells are added to each at a 1:500 or 1:1000 ratio to achieve 70-80% confluency on day of inoculation.
B. 1 or 2 flasks are used for each test setup depending on the number of compounds tested.
C. Maximum passage of 14 generations should be adhered to pending documentation or loss of gene expression.
D. Backup flasks will be maintained in parallel utilizing a lower (5-7) passage generation and kept in different incubator.

INOCULATION:

A. U251-HIF- Decant supernatant from flasks, rinse with 3 ml of PBS remove and add 2 ml of trypsin EDTA to each flask. After incubating several minutes, tap gently to remove cells and place cells in 50ml conical tube. Spin at 1000 rpm for 5 minutes to pellet cells. Decant supernatant and resuspend in 10ml phenol red-free media without G418.
Count using hemocytometer. Calculations are made for 3000 cells/well in 384 well plates.
25 µl of the cell solution is added to each well of the 384 well plates (white flat bottom COSTAR 384 for the luminescence assay).
B. U251 plates are then incubated in 5% CO_2 and 37 ˚ C for 24 hours

FABRICATION OF 384 COMPOUND PLATES:

Proposed Plate Replication Scheme for HIF-1 Assay

1. Plates are shipped to the Natural Products Support Group lab. Plates contain 20µl of a 1mM solution.
2. Plates will replicated on the Apricot 96-tip robotic system (Personal Pipettor 550) as follows:

a. Parent plate will be placed on deck in position 4.
b. 384 well Daughter plates will be placed in a plate stacker.
c. Disposable tips will be put on the Apricot.
d. Isopropanol will be placed in solvent reservoir in position 1.
e. Deep well dilution plate will be placed in position 2.
f. Parent plate will have the barcode read.
g. Apricot will add 390µl of Isopropanol to the dilution deep well plate.
h. Apricot will remove 10µl from the parent plate and move to the dilution plate and mix the solution well (10µl in 400µl).
i. 384 well Daughter plates will be moved to Apricot deck and the barcode read, the first plate will be used to look up in ORACLE for which quadrant to put the plate into, subsequent plates will be checked and assigned.
j. Either 20 or 40µl of this solution will be transferred to the daughter plate.
k. Plates will be re-stacked to a wait drying.
l. Plate maps will be assigned to ORACLE for importing into plate table.

3. Step 2 will be repeated four times for each set of 384 well daughter plates (four parent plates/set of 384 well daughter plates).
4. Daughter plates are dried under vacuum with a residue of 1µl of a 1mM DMSO solution remaining.

V. ADDITION OF COMPOUNDS:

E. Thaw 384 well block drug plate at room temperature and place on shaker on low setting to resuspend.
F. Prepare DPI (Diphenylene iodonium) positive control from frozen stock. DPI is frozen at a stock concentration of 6.3 mM. Working stock solutions are then diluted to a concentration of 2.5µM in phenol red-free media. Two additional dilutions of the working stock are made (1.25 and 0.625µM). These concentrations, when added to test plates (1:2), yield 3 final plate concentrations of 1.25, 0.625 and 0.313 µM respectively. Each 2x concentration is placed in the appropriate Biomek 6 chambered trough according to stage format for the Biomek 2000.
G. Prepare DFX (Desferrioxamine) induction control from frozen stock. Three final working stock concentrations of DFX (80µM, 40µM and 20µM) are made to be added to the HIF- CO_2 plate only (columns 2-4 respectively). This final 1 to 2 dilution in the plate will yield concentrations of 40,20, and 10µM.

H. Phenol red-free media is added to a separate trough for use in the program: all troughs are placed on the Biomek work surface.
I. Along with the troughs, the thawed block compound plate, 2-384 well luminescence plates (U251-HIF-HYPOXIA, and the CO_2) are taken from incubator and are placed on the work surface.
J. Initiate custom designed program sequence, which will begin by adding media to the control (cells only) column of the 384 well plates (See Plate Format). The block plate containing 0.5µl of sample at a concentration of 1000µM is diluted to a concentration of 2µM by adding 249.5µl of media to each sample well using p250 tips. The DFX and DPI control compounds are then added to specific columns of the CO_2 plate.
K. Using p250 tips, the test compounds are added to appropriate 384 well test plates in the following manner: 25µl of the 2µM test concentration are added to each 384 well test plates, as illustrated in plate map format, containing 25µl of U251 cells, creating the final 1µM final test concentration.
L. The CO_2 plate are removed and stored in 5% CO_2, 37˚ C incubator for 24 hours. The hypoxia 384plate is placed in hypoxia chamber and flooded with nitrogen gas for 20 minutes. Resulting in 1-2 % O_2 levels. After 20 minutes chamber is sealed and placed in a 37-degree incubator for 12 to 24 hours (overnight).

VI. READING:

A. If using a fresh bottle of Bright-Glo substrate buffer thaw buffer at room temperature and mix with lyophilized substrate from kit. (See kit instructions) Store at –20 ˚ C
B. If using a bottle that has been stored in the freezer, thaw the bottle using water at room temperature. Approximately 2 hours.
C. Remove CO_2 plate from incubator and hypoxia plates from hypoxia chamber and let equilibrate 1.5 hours at room temperature in 5% CO_2 incubator. (Ice can be added to pan of incubator to help keep temperature around 25-26 ˚ Celsius if necessary).
D. Pour 35ml of the Bright-Glo substrate (Bright-Glo must be no warmer than Room Temperature) into a trough on the work surface, and initiate robotic addition of 40µl Bright Glo to each well of the 384 well luminescence test plates for a total volume of 90µl in each well.
E. Plates are left at room temperature in laminar flow hood for at least 3 minutes and are then read in sequential order using the WALLAC 1420 Reader in Luminescence mode following the custom protocol labeled 100 Milli.
F. Repeat steps D and E for each luminescence plate.

G. Once all Luminescence plates are read, Data is imported into custom EXCEL spreadsheet.
H. Data is then imported into ACCESS database for review.

VI. ANALYSIS

A. The following parameters and definitions are used in analyzing data from the HIF-1 assay.

- Cells Only CO_2
- Cells Only Hypoxic
- Cells & DPI
- Cells & Test Compound

B. Calculated Fields

- Cells Only Induction = Cells Only Hypoxic / Cells Only CO_2
- DPI Induction = DPI / Cells Only CO_2
- Test Induction = Test / Cells Only CO_2
- Fold Induction = Test Induction / Cells Only Induction
- Z Prime = 1 – ((3* Std Cells Only CO_2 + 3* Std Cells Only Hypoxic) / ABS(Avg Cells Only CO_2 – Avg Cells Only Hypoxic))

C. Quality Control

- Surface Maps are generated to evaluate general toxicity profiles. If a surface map demonstrates irregular growth patterns, the plate is flagged for resubmission and confirmation. The purpose is to eliminate potential plate inconsistencies attributed to the plate drying procedures and /or technical errors.

D. Growth (for Diversity, Training, and Titration Retest)

- Cells Only
- Cells & DPI
- Cells & Test Compound

E. Calculated Fields

- Percent Growth = Test / Cells Only * 100