

# An Automated, Cell-based Platform for the Rapid Detection of Novel Androgen Receptor Modulators



Brad Larson<sup>1</sup>, Bruce Sherf<sup>2</sup>, and Peter Banks<sup>1</sup>  
<sup>1</sup>BioTek Instruments, Inc., Winooski, Vermont, USA • <sup>2</sup>INDIGO Biosciences, State College, Pennsylvania, USA



## Introduction

The Androgen Receptor (AR) is a member of the family of nuclear receptors responsive to steroid hormones. AR activity is regulated through the binding of androgenic hormones (e.g., testosterone and di-hydroxy testosterone). In turn, AR functions to regulate the expression of a myriad of genes involved in metabolic processes, cell proliferation/apoptosis, and male sexual differentiation and development. AR dysfunction often has severe health implications, such as androgen insensitivity syndrome and prostate cancer. Consequently, AR commands much attention as a target for the discovery and development of improved drugs. In particular, anti-androgens comprise an important class of drugs used in the treatment prostate cancer.

The aim of the present work was to devise, validate and perform a preliminary (small-scale) automated HTS screening campaign to identify novel modulators of AR activity. The study incorporated a new whole-cell, luciferase-based assay to quantify human Androgen Receptor activity. The performance and specificity of this AR assay were first confirmed using a small commercial library of known nuclear receptor modulators. The validated AR assay was then scaled-up and deployed to screen a library comprising 506 diverse small-molecule compounds of natural origin. The natural compound library was functionally interrogated to identify both activators and inhibitors of AR. Chemicals identified in the primary screen as putative AR inhibitors were further analyzed using a live cell multiplex assay. The multiplexed assay incorporates a fluorescent Calcein-AM assay to monitor viable cell number, as well as a luminescent AR assay, which were performed sequentially in the same assay well. Using this format, cytotoxic compounds that elicited false-positive 'hits' were eliminated from further consideration. Test compounds displaying novel bio-activities to AR, either agonist or antagonist, were further analyzed to establish relative potency values.

Automation of the initial library screens was carried out using an easy-to-use, robust instrumentation. An 8-channel liquid handler was used to prepare serial dilutions of library and reference compounds, followed by their transfer into 384-well assay plates. Subsequent additions of AR reporter cells and detection reagent were performed using a high-speed non-contact reagent dispenser. The small footprint of the instrument, as well as capability to autoclave the dispensing pathway, allowed for sterile manipulations during the assay process. A combination washer/dispenser, capable of media and reagent removal, as well as dispensing cells and other components was then used to perform the live cell multiplex assay. Results demonstrate how the combination of the novel, cell-based chemistries and automation can be used to rapidly, and accurately identify modulators of this important drug target.

## BioTek Instrumentation

### Liquid Handling

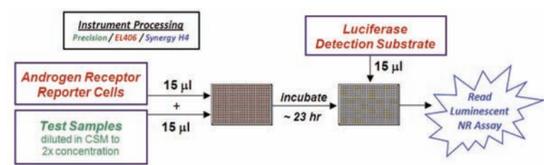
The Precision™ Microplate Pipetting System combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to dilute the library compounds, create compound dose response curves for validation and secondary screening, and transfer all compounds to the 96- and 384-well assay plates.

The EL406™ Combination Washer Dispenser offers fast, accurate media removal and plate washing capabilities through its Dual-Action™ Manifold. It also offers reagent dispensing capabilities through the use of its peristaltic or syringe pumps. The instrument was used for cell dispensing, media removal, buffer wash, as well as LCMA and detection substrate addition in the LCM assay.

### Detection

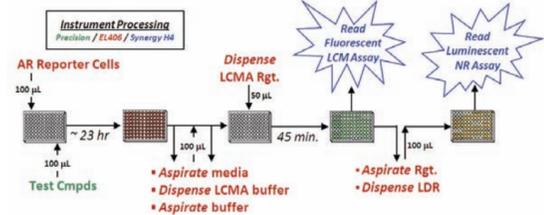
The Synergy™ H4 Hybrid Multi-Mode Microplate Reader combines a filter-based and monochromator-based detection system in the same unit. A dedicated luminescence detection system was used to quantify the luminescent signal from the Androgen Receptor Assay, while the filter-based system was used to detect the fluorescent cell viability signal, which is part of the Live Cell Multiplex Assay.

## INDIGO AR Assay: Primary and Secondary Screening



**Figure 1** – INDIGO Human Androgen Receptor Reporter Assay System. Reporter cells include the luciferase reporter gene functionally linked to an AR-responsive promoter. Therefore modulators of AR activity will lead to an increase or decrease in luminescent output, relative to a basal signal.

Diluted compounds and reporter cells are added to the 384-well assay plates. Following incubation at 37°C/5% CO<sub>2</sub>, detection substrate is then added, and the luminescent signal is quantified.

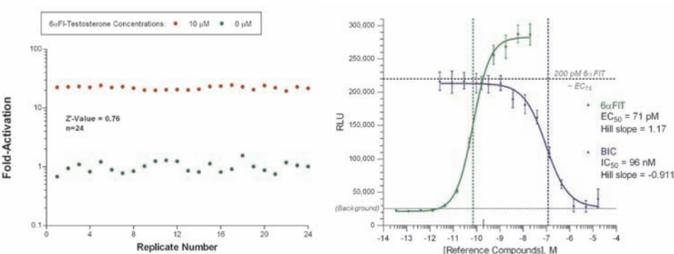


**Figure 2** – INDIGO 96-well format LIVE Cell Multiplex (LCM) Reporter Assay allows secondary screening of test compounds to assess cell toxicity. In addition to the AR reporter system, the assay also utilizes the fluorogenic substrate Calcein-AM (LCMA Reagent) to provide a quantitative measure of the relative number of live cells remaining in the assay wells following exposure to test compounds. In this way, "False-positive" antagonist hits are identified and eliminated from further analyses.

Following cell and compound addition and incubation, media is washed from the wells and the LCMA Reagent is added. The cell-permeable molecule, once taken into the cells, is hydrolyzed to generate the fluorescent molecule Calcein by endogenous esterases found in intact living cells. Following incubation at 37°C/5% CO<sub>2</sub>, the fluorescent signal is quantified. LCMA reagent is then removed from the wells and LDR is added, as with the AR Reporter Assay System. The luminescent signal is then quantified, which is proportional to AR activity in the well.

## Automated AR Assay Validation

A Z'-factor test was performed to validate the automated 384-well AR assay. 6α-FIT was used as the control compound. Twenty-four replicates of 10 μM or 0 μM compound were used as the positive and negative control, respectively. Dose response curves were also created using the control agonist, 6α-Fluoro-Testosterone (6α-FIT), and the control antagonist, Bicalutamide (BIC), to validate the ability of the automated assay to generate correct compound pharmacology.

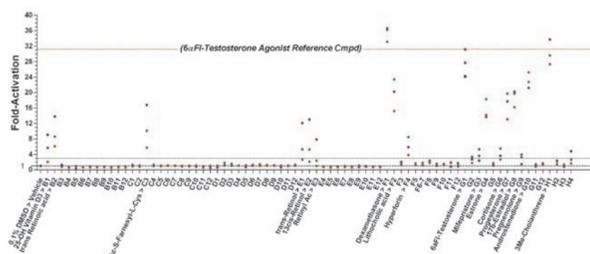


**Figure 3** – Z'-factor validation data. Z' values ≥0.5 are indicative of a robust assay for HTS (Zhang et al., 1999).

**Figure 4** – EC<sub>50</sub> value of 71 pM for 6α-FIT, and IC<sub>50</sub> value of 96 nM for BIC are equivalent to values previously generated using manual methods.

## NR Ligand Library Primary Screen

The 76 member Screen-Well® Nuclear Receptor Ligand Library (generously contributed by ENZO Biochem), containing compounds with known bioactivity to Nuclear Receptors, was used to validate the automated 384-well Human Androgen Receptor Assay setup for screening purposes.

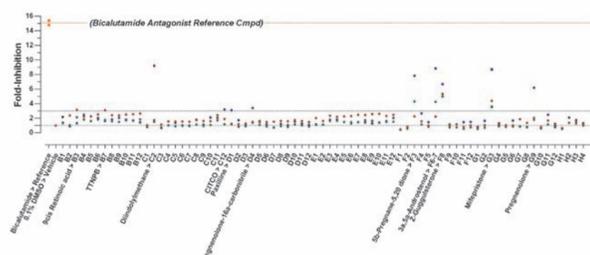


**Figure 5** – Human Androgen Receptor Assay, Agonist Validation Screen using Screen-Well® Nuclear Receptor Ligand Library, BML-2802.

Test compounds were prepared at three sub-concentrations, then screened (n=6) at the following concentrations for agonist activities against AR: • 10 μM, • 2.0 μM, and • 0.40 μM. 0.1% DMSO (•) was included as the vehicle control. 6α-Fluoro-Testosterone, a common reference agonist for AR, is resident within the library (•, Well G1). Fold-activation is defined as [RLU<sub>Test Cmpd</sub> / RLU<sub>Vehicle Control</sub>].

Well location	Test Compound Common Name	Fold-ACTIVATION of Human AR at these concentrations		
		10 μM	2.0 μM	0.40 μM
Vehicle	DMSO, 0.1%	1.00	1.00	1.00
B1	3β-OH-Vitamin D3	8.98	8.71	2.98
B2	Retinoic acid, all-trans	13.8	8.81	6.89
C1	Ac-S-Farnesyl-L-Cysteine	18.8	10.1	6.78
E1	Retinol, all-trans	12.1	8.30	2.84
E2	13-cis-Retinal	13.9	8.37	2.16
E3	Retinyl acetate	7.87	2.40	1.00
F1	Dexamethasone	38.8	38.2	33.1
F2	6α-Fluoro-Testosterone	2.8	2.2	1.2
F3	Lithocholic acid	29.2	23.4	18.3
F4	Hyperforin	8.89	8.49	3.88
G1	6α-Fluoro-Testosterone	24.2	27.7	31.2
		CV = 4.4%	5.8%	4.2%
		Z' = 0.890	0.819	0.882
G3	Mifepristone	2.42	3.83	6.30
G4	Estrone	13.7	18.3	14.2
G6	Carbonyl	3.83	8.83	3.71
G7	Progesterone	17.7	19.7	13.1
G8	17β-Estradiol	20.2	19.7	16.2
G9	Pregnenolone	1.47	3.28	3.88
G10	Androstenedione	21.2	22.7	35.2
H1	3-Me-Cholanthrene	33.7	29.8	27.3

**Known BioActivities**  
 Synthetic retinoids of vitamin A: major circulating form of vitamin D; VDR agonist  
 Retinoid agonist and agonist for the estrogen receptor  
 Vitamin A, RAR beta agonist  
 Synthetic retinoids possess an effect in regulating 11α-oxo androstane levels  
 Vitamin A: active a natural form of vitamin A  
 GR agonist with anti-inflammatory activity  
 Natural ligand for VDR and the RXR; activates VDR without raising calcium levels  
 Activator of the pregnane X-receptor (PXR); key regulator of CYP3A4 transcription  
 Common Reference Agonist for Androgen Receptor (AR)  
 Antagonist at progesterone (PR) and glucocorticoid (GR) receptors  
 Inverse agonist and agonist for the estrogen receptor  
 Agonist glucocorticoid receptor using stress increases glucocorticoids  
 Steroidogenic progesterone receptor (PR) agonist  
 Steroidogenic estrogen receptor (ER) agonist  
 PR Ligand; Cytosolic PRF11 inducer  
 Receptor to testosterone and estradiol; weak selective receptor modulator for AR  
 AR hydrotrope receptor (ARH) agonist



**Figure 6** – Human Androgen Receptor Assay, Antagonist Validation Screen using Screen-Well® Nuclear Receptor Ligand Library, BML-2802.

Test compounds were prepared at three sub-concentrations, then screened (n=6) at the following concentrations for antagonist activities: • 10 μM, • 2.0 μM, and • 0.40 μM. AR Reporter cells were pre-treated with 200 pM (~EC<sub>75</sub>) of 6α-Fluoro-Testosterone as the challenge agonist. 0.1% DMSO (•, vehicle) and 5.0 μM Bicalutamide (•, Reference antagonist) were tested as controls. Mifepristone, another common reference antagonist for AR, is resident within the library (•, Well G3). Fold-inhibition is defined as [RLU<sub>Vehicle Cmpd</sub> / RLU<sub>Test Control</sub>].

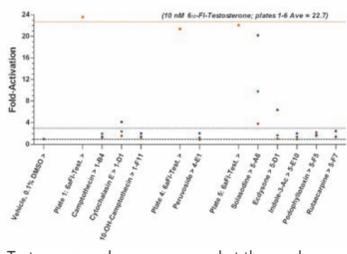
Plate 1 Well	Test Compound Common Name	Fold-INHIBITION of Human AR at these concentrations		
		10 μM	2.0 μM	0.40 μM
Vehicle	DMSO, 0.1%	1.00	1.00	1.00
		CV = 6.4%	6.6%	6.6%
		Z' = 0.782	0.799	0.781
Reference Antagonist	Bicalutamide, 5.0 μM	15.4	14.8	20.3
F3	5β-Pregnen-3,20-dione	7.82	4.29	2.25
F6-7	3α, 5α-Androstano	8.82	4.23	2.22
F8	Z-Guggulsterone	6.89	5.00	5.36
G3	Mifepristone	8.71	3.88	4.39

**Known BioActivities**  
 Non-steroidal androgen receptor antagonist; common Reference Antagonist for AR  
 Converted to progesterone via Progesterone 5α-Reductase  
 Inverse agonist of the murine constitutive androstane receptor; CARβ  
 Broad spectrum steroid receptor ligand; SR, PR, GR & FXR antagonists  
 Antagonist of progesterone (PR) and glucocorticoid (GR) receptors

The luminescent signal from library compounds was compared to those generated from vehicle control wells containing 0.1% DMSO only, to generate fold-activation and fold-inhibition values. Hits were defined as compounds having fold values ≥3 for two of the three concentrations tested. Results from known AR agonists and antagonists demonstrate the utility of the automated 384-well Human Androgen Receptor Assay to accurately detect AR modulators.

## Natural Product Library Primary Screen

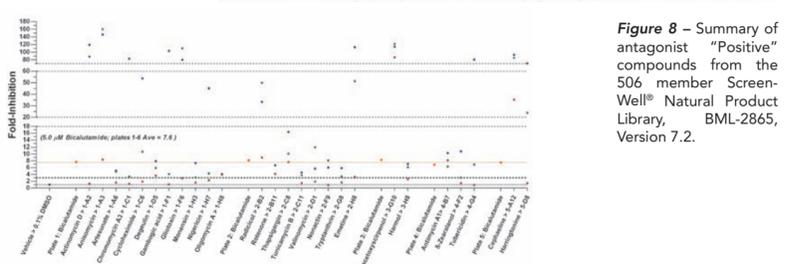
The 506 compound Screen-Well® Natural Product Library (generously contributed by ENZO Biochem) was then screened using the automated 384-well Human Androgen Receptor Assay. The library was chosen to examine whether novel AR modulators could be identified within the compound set.



**Figure 7** – Summary of agonist "Positive" compounds from the 506 member Screen-Well® Natural Product Library, BML-2865, Version 7.2.

Test compounds were prepared at three sub-concentrations, then screened (n=1) at the following concentrations for agonist activities: • 1:1,000, • 1:5,000 and • 1:25,000. 0.1% DMSO (•, vehicle) and 10 nM 6α-Fluoro-Testosterone (•, Reference agonist) were added to each of the six 384-well assay plates.

Library Plate	Well Location	Test Compound Common Name	Highest Conc. Tested (μM)	Fold-ACTIVATION of AR at these dilutions		
				1:1,000	1:5,000	1:25,000
Plates 1-6	Vehicle	DMSO	0.10%	1.00	--	--
Plate 1	Reference Agonist	6α-Fluoro-Testosterone	10.0 nM	23.6	CV = 8.9 %	
		Cytoschalasin E	4.04 μM	4.10	2.42	1.60
					Z' = 3.20	3.96
Plate 5	Reference Agonist	6α-Fluoro-Testosterone	10.0 nM	22.1	CV = 6.7 %	
		Solasodine	4.84 μM	20.2	9.76	3.77



**Figure 8** – Summary of antagonist "Positive" compounds from the 506 member Screen-Well® Natural Product Library, BML-2865, Version 7.2.

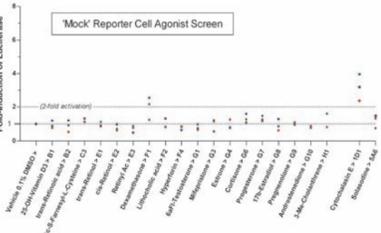
Test compounds were prepared at three sub-concentrations, then screened (n=1) at the following concentrations for antagonist activities: • 1:1,000, • 1:5,000 and • 1:25,000. AR Reporter cells were pre-treated with 200 pM (~EC<sub>75</sub>) of 6α-Fluoro-Testosterone as the challenge agonist. 0.1% DMSO (•, vehicle) and 5.0 μM Bicalutamide (•, Reference agonist) were added to each of the six 384-well assay plates.

Library Plate	Well Location	Test Compound Common Name	Highest Conc. Tested (μM)	Fold-INHIBITION of AR at these dilutions		
				1:1,000	1:5,000	1:25,000
Plates 1-6	Vehicle	DMSO	0.10%	1.00	--	--
Plate 1	Reference Agonist	Bicalutamide	5.0 μM	7.70	CV = 7.6 %	
		Actinomycin D	4.28 μM	119	89.2	132
		Actinomycin D3	294 μM	140	100	131
		Actinomycin D4	1.59 μM	5.03	4.03	182
		Chromomycin A3	183 μM	82.8	3.38	124
		Cycloheximide	236 μM	183	187	180
		Doxorubicin	187 μM	7.02	5.92	3.95
		Genistein	3.9 μM	10.3	4.11	188
		Quercetin	4.28 μM	4.89	3.07	288
		Resveratrol	239 μM	6.8	7.07	3.05
		Resveratrol	4.28 μM	45.1	4.29	232
		Silvestrol	280 μM	45.1	4.29	232
		Silvestrol	4.28 μM	8.71	3.04	6.14

**Known BioActivities**  
 Non-steroidal androgen receptor antagonist; common Reference Antagonist for AR  
 DNA intercalating agent and transcription inhibitor; induces apoptosis  
 Antitumor nucleoside triphosphate; inhibits pyridyl transferases; an indirect agonist  
 CDCC2 inhibitor; displays antiproliferative action against BR tumor cell lines  
 Chromomycin A3 is a GC-specific DNA ligand which inhibits transcription  
 Inhibits protein synthesis in both prokaryotes and eukaryotes  
 Inhibits cytoprotection of EDCO/2 expression; inhibitor of AR; may induce apoptosis  
 Apoptosis induction proceeds via upregulation of bax and inhibition of bcl-2 expression  
 Blocks membrane fluidity; induces Ca<sup>2+</sup> release from liver or brain mitochondria  
 No transport; blocks glycerol uptake; inhibits proliferation through cell cycle arrest and apoptosis  
 Chloroquinone member; stimulates Ca<sup>2+</sup> release from mitochondrial stores; blocks Golgi function; inhibits mitochondrial ATPases; stimulates pyruvate oxidation; induces apoptosis

## Hit Confirmation and Secondary Screening

Compounds demonstrating positive AR agonist or antagonist activity were confirmed using two different methods. Positive agonists were further tested using "Mock" reporter cells, which contained the luciferase reporter gene construct without expressed Androgen Receptor. In this way, compounds which induced luciferase expression in the absence of receptor activation could be removed from further consideration.



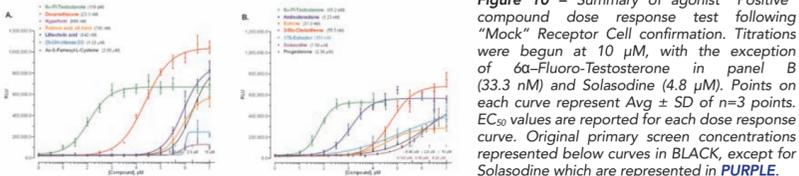
**Figure 9** – Secondary screen of Agonist-Positive compounds against "Mock" Reporter Cells. Screen-Well® Nuclear Receptor Ligand Library, BML-2802 (• 1 μM, • 2.0 μM, • 0.40 μM) Screen-Well® Natural Product Library BML-2865 Version 7.2 (• 1:1,000, • 1:5,000, • 1:25,000)

Putative "positive" antagonists were further evaluated using INDIGO's Live Cell Multiplex (LCM) Assay. A compounds were tested at their highest HTS assay concentrations.

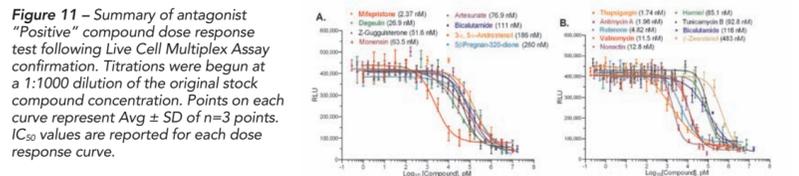
Library Plate	Well Location	Test Compound Common Name	Highest Conc. Tested (μM)	Fold-INHIBITION of AR at these dilutions		
				1:1,000	1:5,000	1:25,000
Plates 1-6	Vehicle	DMSO	0.10%	1.00	--	--
Plate 1	Reference Agonist	6α-Fluoro-Testosterone	10.0 nM	23.6	CV = 8.9 %	
		Cytoschalasin E	4.04 μM	4.10	2.42	1.60
					Z' = 3.20	3.96
Plate 5	Reference Agonist	6α-Fluoro-Testosterone	10.0 nM	22.1	CV = 6.7 %	
		Solasodine	4.84 μM	20.2	9.76	3.77

Compounds yielding greater than 90% Live Cells at the assay endpoint are scored as true inhibitors of AR activity. Compounds yielding between 90-50% Live Cells may be cytostatic, and are included in additional analyses. Compounds yielding <50% Live Cells are cyto-toxic, scored as "false positive" antagonists, and removed from further testing.

Next, fill dose-response curves were generated using the confirmed positive AR agonists and antagonists to determine EC<sub>50</sub> or IC<sub>50</sub> values.



**Figure 10** – Summary of agonist "Positive" compound dose response test following "Mock" Receptor Cell confirmation. Titrations were begun at 10 μM, with the exception of 6α-Fluoro-Testosterone in panel B (33.3 nM) and Solasodine (4.8 μM). Points on each curve represent Avg ± SD of n=3 points. EC<sub>50</sub> values are reported for each dose response curve. Original primary screen concentrations represented below curves in BLACK, except for Solasodine which are represented in PURPLE.



**Figure 11** – Summary of antagonist "Positive" compound dose response test following Live Cell Multiplex Assay confirmation. Titrations were begun at a 1:1000 dilution of the original stock compound concentration. Points on each curve represent Avg ± SD of n=3 points. IC<sub>50</sub> values are reported for each dose response curve.

## Conclusions

- The cell-based Human Androgen Receptor Assay from INDIGO provides a streamlined, robust HTS assay
- INDIGO's Live Cell Multiplex Assay is an efficient method to assess cytotoxic properties of test compounds while confirming their AR-inhibitory activity
- Cryopreserved reporter cells further simplify the assay procedure and provide increased repeatability during the primary and secondary screening process
- The BioTek Precision and EL406 are able to easily titrate and transfer library compounds, dispense cells and reagents, and perform the media and buffer removals necessary during the LCM assay
- BioTek's Synergy H4 is capable of detecting the luminescent and fluorescent signals from each of the assays, without the need to change the optical setup of the instrument
- This combination of AR assay and instrumentation provides an ideal platform for primary HTS and secondary screening of library test compounds